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<b>(21) International Application Number:</b> PCT/US98/16417 <b>(22) International Filing Date:</b> 5 August 1998 (05.08.98)  <b>(30) Priority Data:</b> 08/910,973 7 August 1997 (07.08.97) US  <b>(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application</b> US 08/910,973 (CIP) Filed on 7 August 1997 (07.08.97)  <b>(71) Applicant (for all designated States except US):</b> FRED HUTCHINSON CANCER RESEARCH CENTER [US/US]; 1124 Columbia Street, Seattle, WA 98104 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> TAPSCOTT, Stephen, J. [US/US]; 3419 S. Dose Terrace, Seattle, WA 98144 (US). OLSON, James, M. [US/US]; 4733 Lake Washington Boulevard S., Seattle, WA 98118 (US).  <b>(74) Agent:</b> SHEINESS, Diana, K.; Christensen O'Connor Johnson & Kindness PLLC, Suite 2800, 1420 Fifth Avenue, Seattle, WA 98101 (US).		<b>(81) Designated States:</b> AU, CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> EXPRESSION OF NEUROGENIC bHLH GENES IN PRIMITIVE NEUROECTODERMAL TUMORS  <b>(57) Abstract</b>  Provided are methods of classifying and prognosticating human neuroectodermal tumors by analyzing a sample of the tumor to determine whether various bHLH proteins are detectably expressed in the sample. In the methods disclosed herein, expression of the bHLH genes is assessed by measuring transcripts or proteins expressed from the genes.		

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## EXPRESSION OF NEUROGENIC bHLH GENES IN PRIMITIVE NEUROECTODERMAL TUMORS

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This application is a continuation-in-part of PCT application No. PCT/US96/17532, filed October 30, 1996, which is a continuation-in-part of U.S. application No. 08/552,142, filed November 2, 1995, which is a continuation-in-part of PCT application No. PCT/US95/05741, filed May 8, 1995, which is a continuation-in-part of parent application U.S. Serial No. 08/239,238, filed May 6, 1994 (abandoned).

### Field of the Invention

The invention relates to molecular biology and in particular to genes and proteins involved in vertebrate neural development and to methods for classifying and prognosticating neuroectodermal tumors.

### Background of the Invention

Transcription factors of the basic-helix-loop-helix (bHLH) family are implicated in the regulation of differentiation in a wide variety of cell types, including trophoblast cells (Cross et al., *Development* 121:2513-2523, 1995), pigment cells (Steingrimsson et al., *Nature Gen.* 8:251-255, 1994), B-cells (Shen, C.P. and T. Kadesch., *Molec. & Cell. Biol.* 15:3813-3822, 1995; Zhuang et al., *Cell* 79:875-884, 1994), chondrocytes and osteoblasts (Cserjesi et al., *Development* 121:1099-1110, 1995; Tamura, M. and M. Noda., *J. Cell Biol.* 126:773-782, 1994), and cardiac muscle (Burgess et al., *Develop. Biol.* 168:296-306, 1995; Hollenberg et al., *Molec.*

& *Cell. Biol.* 15:3813-3822, 1995). bHLH proteins form homodimeric and heterodimeric complexes that bind with DNA in the 5' regulatory regions of genes controlling expression.

Perhaps the most extensively studied sub-families of bHLH proteins are those that regulate myogenesis and neurogenesis. The myogenic bHLH factors, (MyoD, myogenin, Myf5, and MRF4), appear to have unique as well as redundant functions during myogenesis (Weintraub, H., *Cell* 75:1241-1244, 1993; Weintraub et al., *Science* 251:761-766, 1991). It is thought that either Myf5 or MyoD is necessary to determine myogenic fate, whereas myogenin is necessary for events involved in terminal differentiation (Hasty et al., *Nature* 364:501-506, 1993; Nabeshima et al., *Nature* 364:532-535, 1993; Rudnicki et al., *Cell* 75:1351-1359, 1993; Venuti et al., *J. Cell Biol.* 128:563-576, 1995). Moreover, Myf expression has been observed in a number of rhabdomyosarcomas, and has been proposed as a marker for that category of tumor (Clark et al., *Br. J. Cancer* 64:1039-1042, 1991).

Recent work on neurogenic bHLH proteins suggests parallels between the myogenic and neurogenic sub-families of bHLH proteins. Genes of the *Drosophila melanogaster achaete-scute* complex and the *atonal* gene have been shown to be involved in neural cell fate determination (Anderson, D. J., *Cur. Biol.* 5:1235-1238, 1995; Campuzano, S. and J. Modolell., *Trends in Genetics* 8:202-208, 1992; Jaman et al., *Cell* 73:1307-1321, 1993), and the mammalian homologs, *MASH1* and *MATH1*, are expressed in the neural tube at the time of neurogenesis (Akazawa et al., *J. Biol. Chem.* 270:8730-8738, 1995; Lo et al., *Genes & Dev.* 5:1524-1537, 1991). Two related vertebrate bHLH proteins, neuroD1 and NEX-1/MATH-2, are expressed slightly later in CNS development, predominantly in the marginal layer of the neural tube and persisting in the mature nervous system (Bartholoma, A. and K. A. Nave, *Mech. Dev.* 48:217-228, 1994; Lee et al., *Science* 268:836-844, 1995; Shimizu et al., *Eur. J. Biochem.* 229:239-248, 1995). NeuroD1 was also cloned as a factor that regulates insulin transcription in pancreatic beta cells and named "Beta2" (Naya et al., *Genes & Dev.* 9:1009-1019, 1995). Constitutive expression of neuroD1 in developing *Xenopus* embryos produces ectopic neurogenesis in the ectodermal cells, indicating that neuroD genes are capable of regulating a neurogenic program. A neuroD1 homolog having 36,873 nucleotides has been identified in *C. elegans* (Lee et al., 1995; Genbank Accession No. 010402), suggesting that this molecular mechanism of regulating neurogenesis may be conserved between vertebrates and invertebrates.

A human *achaete-scute* homolog has been identified and cloned whose predicted protein of 238 amino acids is 95% homologous to the mouse hash1 gene (Ball et al., *Proc. Natl. Acad. Sci. USA* 90:5648-5652, 1993). Northern blots revealed that transcripts from this bHLH gene were detectable in two types of cancer  
5 with neuroendocrine features, namely small cell lung cancer, and the calcitonin-secreting medullary thyroid carcinoma (*id.*). Thus, hash1 was proposed to provide a marker for cancers with neuroendocrine features.

Primitive neuroectodermal tumors (PNET) are the most common of the malignant central nervous system tumors that occur in children (Biegel et al., *Genes, Chrom. & Cancer* 14: 85-96, 1995). Both supratentorial and infratentorial PNETs occur. Medulloblastoma, an infratentorial tumor that expresses neuronal intermediate filaments, synaptic vesicle proteins, growth factor receptors, and adhesion molecules, is the prototypic PNET. The location (posterior fossa) and properties of medulloblastomas suggests that they arise from neuroblasts that escape terminal  
15 differentiation (Trojanowski, J.Q., et al. *Mol. Chem. Neuropathol.* 17:121-135, 1992). Abnormalities in chromosome 17 have been observed in PNET biopsies, though the significance of these abnormalities has not been determined (Biegel et al., 1995; Schultz, et al., *Genes, Chrom. & Cancer* 16:196-203, 1996). Factors presently used to classify and prognosticate brain tumors include location, histopathology,  
20 patient age, and biological behavior of the tumor. However, such bases for tumor identification are not always sufficient for accurate prognostication. Supratentorial and infratentorial PNETs cannot always be distinguished, though they may respond differently to therapy. (Heideman, R.L. et al. "Tumors of the central nervous system." In: P.A. Pizzo and D.G. Poplack (eds.), *Principles and Practice of Pediatric Oncology*, 2nd. ed., pp. 633-682, 1993; Rorke, L.B., et al. *Cancer* 56:1869-1886, 1996; Packer, R.J., et al. *J. Neurosurg.* 81:690-698, 1994; Cohen, B.H. et al. *J. Clin. Oncol.* 13:1687-1696, 1995).  
25

Because different types of brain tumors respond differently to various therapeutic regimens, accurate classification is highly useful to the physician in  
30 determining the best course of treatment for a particular patient. Medulloblastomas about half of the time are confined to the bridge that connects the two halves of the cerebellum (vermis). Medulloblastoma often is an aggressive and highly malignant type of tumor, and may invade other portions of the brain and spinal column. Thus, diagnosis based on location is not always reliable. Histologically, medulloblastoma is  
35 highly cellular, consisting of undifferentiated small dark round cells. Other PNETs

with an identical histologic appearance, occurring predominantly in infants, are found at other locations in the brain. Thus, markers for a more accurate determination of PNET origin would facilitate the assignment of the therapeutic regimen most likely to be effective. Currently, no effective biologic markers exist for facilitating the stratification of treatment groups, assisting in prognosis, or providing targets for therapeutic intervention (Heideman et al., 1993).

#### Summary of the Invention

The presently disclosed neuroD proteins represent a new sub-family of bHLH proteins and are implicated in vertebrate neuronal, endocrine and gastrointestinal development. Mammalian and amphibian neuroD proteins have been identified, and polynucleotide molecules encoding neuroD proteins have been isolated and sequenced. NeuroD genes encode proteins that are distinctive members of the bHLH family. In addition, the present invention provides a family of neuroD proteins that share a highly conserved HLH region. Representative polynucleotide molecules encoding members of the neuroD family include neuroD1, neuroD2 and neuroD3.

A representative nucleotide sequence encoding murine neuroD1 is shown in SEQ ID NO:1. The HLH coding domain of murine neuroD1 resides between nucleotides 577 and 696 in SEQ ID NO:1. The deduced amino acid sequence of murine neuroD1 is shown in SEQ ID NO:2. There is a highly conserved region following the helix-2 domain from amino acid 150 through amino acid 199 of SEQ ID NO:2 that is not shared by other bHLH proteins.

A representative nucleotide sequence encoding *Xenopus* neuroD1 is shown in SEQ ID NO:3. The HLH coding domain of *Xenopus* neuroD1 resides between nucleotides 376 and 495 in SEQ ID NO:3. The deduced amino acid sequence of *Xenopus* neuroD1 is shown in SEQ ID NO:4. There is a highly conserved region following the helix-2 domain from amino acid 157 through amino acid 199 of SEQ ID NO:4 that is not shared by other bHLH proteins.

Representative nucleotide and deduced amino acid sequences of the human neuroD family are shown in SEQ ID NOS:8-15. Representative nucleotide and deduced amino acid sequences of a human homolog of murine neuroD1 are shown in SEQ ID NOS:8 and 9 (partial genomic sequence) and SEQ ID NOS:14 and 15 (human neuroD1 cDNA). Representative nucleotide and deduced amino acid sequences of the human and murine neuroD2 are shown in SEQ ID NOS:10 and 11, and 16 and 17, respectively. Representative nucleotide and deduced amino acid sequences for human neuroD3 are shown in SEQ ID NOS:12 and 13. The disclosed

human clones, 9F1 (and its corresponding cDNA HC2A; now referred to as human neuroD1) and 14B1 (now referred to as human neuroD2), have an identical HLH motif: Amino acid residues 117-156 in SEQ ID NO:9 and 15, and residues 137-176 in SEQ ID NO:11 (corresponding to nucleotides 405-524 of SEQ ID NO:8 and  
5 SEQ ID NO:14, and nucleotides 463-582 of SEQ ID NO:10). Comparison of the deduced amino acid sequences of these neuroD genes shows that human neuroD3 contains an HLH domain between amino acid residues 108-147 of SEQ ID NO:13 (corresponding to nucleotides 376-495 of SEQ ID NO:12) and that murine neuroD2 contains an HLH domain between amino acids residues 138-177 of SEQ ID NO:17  
10 (corresponding to nucleotides 641-760 of SEQ ID NO:16). The HLH domain of murine neuroD2 is identical to that of the human neuroD1 and human neuroD2 proteins. Similar analyses indicated that mouse neuroD3 contains an HLH domain between amino acid residues 109-148 of SEQ ID NO:22 (corresponding to nucleotides 425-544 of SEQ ID NO:21).

15 Expression of several bHLH genes were analyzed in cerebellar and cerebral primitive neuroectodermal tumors (PNETs), gliomas, and in cell lines derived from a variety of neuroectodermal tumors. Generally, the observed patterns of neuroD expression distinguished subclasses of neuroectodermal tumors and generally recapitulated gene expression patterns of tissues from which neuroectodermal tumors  
20 arise. For example, a striking association of neuroD3 with aggressive cases of medulloblastoma was noted, suggesting that neuroD3 provides a useful prognosticator for aggressive medulloblastoma.

#### Detailed Description of the Preferred Embodiment

The subject invention provides three representative members of the neuroD  
25 family of genes, namely, neuroD1, neuroD2 and neuroD3 (also called "neurogenin"). More specifically, the invention provides murine neuroD1 (SEQ ID NOS:1 and 2, *Xenopus laevis* neuroD1 (SEQ ID NOS:3 and 4, human neuroD1 (SEQ ID NOS:8, 9, 14, and 15, human neuroD2 (SEQ ID NOS:10 and 11), human neuroD3 (SEQ ID NOS:12 and 13), murine neuroD2 (SEQ ID NOS:16 and 17), and murine neuroD3  
30 (SEQ ID NOS:21 and 22).

Provided are methods of classifying human neuroectodermal tumors by analyzing a sample of the tumor, such as, for example, a biopsy or a sample of an excised tumor. For this analysis, the expression of at least one basic helix loop (bHLH) gene is measured in the sample, and the tumor is classified as belonging to a  
35 particular subclass of neuroectodermal tumor in accord with the observed expression

pattern. Examples of neuroectodermal tumor subclasses amenable to this analysis include supratentorial PNETs, such as neuroblastoma, as well as infratentorial PNETs, such as medulloblastoma. Predetermined profiles of bHLH expression associated with particular subclasses of neuroectodermal tumors are established by  
5 collecting tumor samples that have been classified by conventional methods, analyzing them for bHLH expression, and correlating the observed patterns of expression with the various subclasses of tumor. It is demonstrated that bHLH expression in several instances correlates with tumor subclass, and it is contemplated that these methods will be applicable to additional subclasses of neuroectodermal tumors. NeuroD genes  
10 whose measurement is useful in this context include neuroD1, neuroD2, neuroD3, but it is contemplated that additional neuroD family members will also contribute to this method of tumor classification. Hence, at least one, and preferably several, bHLH genes are analyzed in each tumor sample. For example, individual tumor samples may be analyzed for the expression of neuroD1, neuroD2, and neuroD3. It is determined  
15 that the tumor belongs to a given subclass of neuroectodermal tumors if the bHLH gene or genes expressed in the sample corresponds to a predetermined profile of basic helix loop helix expression associated with that subclass of neuroectodermal tumor.

Tumor samples typically will be obtained during excision of the tumor from the patient, but may be obtained by other means, such as a biopsy or a spinal tap.  
20 Alternatively, the tumor sample may be obtained from the site of a metastatic tumor that originated from a neural tumor but that is located outside the primary site of disease. Such metastatic tumors may appear in any part of the body, but most often are found in the spinal cord. Tumor samples also may be obtained from spinal fluid, which may be analyzed directly, or may be subjected to centrifugation to collect  
25 cellular material which in turn is analyzed.

The term "bHLH expression" refers to expression of the bHLH gene in the form of transcripts and/or polypeptide products. bHLH expression thus can be measured by using assays that detect bHLH RNA or polypeptides. bHLH transcripts typically are measured by amplification using polymerase chain reaction (PCR), or  
30 hybridization under stringent conditions of RNA from the tumor sample with DNA or RNA probes corresponding specifically to the nucleotide sequences of non-conserved regions of cloned bHLH cDNAs, genes, or subportions thereof. By "non-conserved region" is meant that the probe does not encode the conserved bHLH domain itself. Hybridization methods for detecting bHLH transcripts include hybridization in  
35 solution, Northern blot analysis, dot blot or slot blot analysis, *in situ* hybridization,



hybridizations wherein the probe is anchored to a solid substratum, liquid hybridization wherein the formed duplexes are subsequently captured on a solid substratum, or other methods of hybridization. Probes may be labeled directly, e.g., with radioisotopes or biotin, or may contain nucleotide sequences complementary to a secondary probe that itself is labeled.

The term "capable of hybridizing under stringent conditions" means that the probe anneals under stringent hybridization conditions to the target nucleic acid molecule. Individual probe molecules are generally between 7-1000 nucleotides in length, and preferably are between 10 and 650 nucleotides in length. Probe molecules longer than 1000 nucleotides may be used, but it is preferable that these be sheared to fragments of 200-500 nucleotides in length prior to use. Long DNA molecules to be used as probes may be sheared mechanically, enzymatically, or by brief alkali treatment.

"Stringent hybridization" is generally understood in the art to mean that the nucleic acid duplexes that form during the hybridization reaction are perfectly matched or nearly perfectly matched. Several rules governing nucleic acid hybridization have been well established. For example, it is standard practice to achieve stringent hybridization for polynucleotide molecules >200 nucleotides in length by hybridizing at a temperature 15°-25°C below the melting temperature ( $T_m$ ) of the expected duplex, and 5°-10°C below the  $T_m$  for oligonucleotide probes (e.g., Sambrook et al., *Molecular Cloning*, [2d ed.], Cold Spring Harbor Laboratory Press, 1989, which is hereby incorporated by reference; see Section 11.45). Under such conditions, stable hybrid duplexes will form only if few or no mismatches are present. When Northern or Southern blots are performed, the detection of only well-matched hybrids can be assured by conducting the hybridization step under low or moderate stringency conditions, and then conducting the final wash steps under stringent wash conditions, i.e., at about 10-14°C below the  $T_m$ .

The  $T_m$  of a nucleic acid duplex can be calculated using a formula based on the % G+C contained in the nucleic acids, and that takes chain length into account, such as the formula  $T_m = 81.5 - 16.6 (\log [Na^+]) + 0.41 (\% G+C) - (600/N)$ , where  $N$  = chain length (Sambrook et al., 1989, at Section 11.46). It is apparent from this formula that the effects of chain length on  $T_m$  is significant only when rather short nucleic acids are hybridized, and also that the length effect is negligible for nucleic acids longer than a few hundred bases.

The term "capable of hybridizing under stringent conditions" as used herein means that the subject nucleic acid molecules (whether DNA or RNA) anneal under stringent hybridization conditions to an oligonucleotide probe of 15 or more contiguous nucleotides of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, SEQ ID NO:16, or SEQ ID NO:21. Oligonucleotides 15 nucleotides or more in length are extremely unlikely to be represented more than once in a mammalian genome, hence such oligonucleotides can form specific hybrids (see, for example, Sambrook et al., at Section 11.7).

The choice of hybridization conditions will be evident to one skilled in the art and will generally be guided by the purpose of the hybridization, the type of hybridization (DNA-DNA or DNA-RNA), and the level of desired relatedness between the sequences. See, for example: Sambrook et al., 1989.; Hames and Higgins, eds., *Nucleic Acid Hybridization, A Practical Approach*, IRL Press, Washington DC, 1985; Berger and Kimmel, eds., *Methods in Enzymology, Vol. 52, Guide to Molecular Cloning Techniques*, Academic Press Inc., New York, NY, 1987; and Bothwell, Yancopoulos and Alt, eds., *Methods for Cloning and Analysis of Eukaryotic Genes*, Jones and Bartlett Publishers, Boston, MA 1990; which are incorporated by reference herein in their entirety. The stability of nucleic acid duplexes is known to decrease with an increased number of mismatched bases, and further to be decreased to a greater or lesser degree depending on the relative positions of mismatches in the hybrid duplexes. Thus, the stringency of hybridization may be used to maximize or minimize the stability of such duplexes. Hybridization stringency can be altered by: adjusting the temperature of hybridization; adjusting the percentage of helix-destabilizing agents, such as formamide, in the hybridization mix; and adjusting the temperature and/or salt concentration of the wash solutions. For filter hybridizations, the final stringency of hybridization often is determined by the salt concentration and/or temperature used for the post-hybridization washes. In general, the stringency of hybridization reaction itself may be reduced by reducing the percentage of formamide in the hybridization solution. High stringency conditions, for example, may involve high temperature hybridization (e.g., 65-68°C in aqueous solution containing 4-6 X SSC (1 X SSC = 0.15 M NaCl, 0.015 M sodium citrate), or 42°C in 50% formamide combined with washes at high temperature (e.g., 5-25°C below the T<sub>m</sub>), in a solution having a low salt concentration (e.g., 0.1 X SSC). Low stringency conditions may involve lower hybridization temperatures (e.g., 35-42°C in 20-50% formamide) with washes conducted at an intermediate temperature (e.g., 40-

60°C) and in a wash solution having a higher salt concentration (e.g., 2-6 X SSC). Moderate stringency conditions, which may involve hybridization in 0.2-0.3M NaCl at a temperature between 50°C and 65°C and washes in 0.1 X SSC, 0.1% SDS at between 50°C and 55°C, may be used in conjunction with the disclosed  
5 polynucleotide molecules as probes to identify genomic or cDNA clones encoding members of the neuroD family.

To measure the amount of bHLH protein in a sample, antibody-based methods can be used. For example, either monoclonal or polyclonal antibody directed against the target protein can be used in Western blots, radioimmunoassays (RIA), enzyme-  
10 linked immunosorbent assays (ELISA), or the like.

For purposes of the subject invention, bHLH expression is "detected" if the level of bHLH transcripts or protein is elevated above the background level in the assay that is conducted to measure the transcripts or protein. "Background level" is the level of signal observed in control reactions in which the target transcript or  
15 protein is not present.

In one embodiment of the invention, a method is provided wherein a human neuroectodermal tumor is classified as a medulloblastoma by measuring neuroD1 and neuroD3 expression in a sample of the tumor, and determining that the tumor is a medulloblastoma if both neuroD1 and neuroD3 expression are detected in the sample.

20 High level expression of neuroD in neuroendocrine tumors and in rapidly proliferating regions of embryonic neural development (see below) indicates that measuring the levels of expression of neuroD or other bHLH genes may provide prognostic markers for assessing the growth rate and invasiveness of a neural tumor. Provided here is a method for prognosticating a human medulloblastoma based on  
25 measuring the expression of neuroD3, which is expressed in some but not all medulloblastomas (see Table 1, Example 17). It is determined that the tumor is an aggressive medulloblastoma if neuroD3 expression above the background level is detected in the sample. By "prognosticating" is meant the foretelling of the probable course of a tumor in advance of treatment, and predicting the likelihood that  
30 treatment will be successful.

The instant invention may also be used in the identification of sites of metastases. The methods of the invention may be used, for example, to identify cells located outside the primary site (e.g., located in a lymph node or in the bone marrow) that are expressing a constellation of neuroD family members consistent with a tumor  
35 from which they are suspected to have originated. The identification of such cells

would indicate the presence of metastatic disease. The methods of the invention may also be used similarly to assay for microscopic disease in bone marrow or peripheral blood stem cells harvested for autologous marrow transplant or stem cell re-infusion. In addition, the methods may be used to determine the degree of tumor cell reduction  
5 in marrow or stem cells that have undergone purging to eliminate tumor cells or that have undergone physical selection away from tumor cells.

The neuroD family of genes function during the development of the nervous system. Like *MATH1* (Lo et al., *Genes & Dev.* 5:1524-1537, 1991), the expression of neuroD3 peaks during embryonic development and is not detected in the mature  
10 nervous system. NeuroD2 shows a high degree of sequence similarity to both neuroD1 and *NEX-1/MATH2*, and is similarly expressed both during embryogenesis and in the mature nervous system, demonstrating an expression pattern that partially overlaps with neuroD1. Like neuroD1, neuroD2 when expressed by transfection in *Xenopus* embryos induces neurogenesis in ectodermal cells. Heterologous expression  
15 of neuroD1 and neuroD2 indicates that these highly similar transcription factors demonstrate some target specificity, with the *GAP-43* promoter being activated by neuroD2 and not by neuroD1. The partially overlapping expression pattern and target specificity of neuroD1 and neuroD2 suggests that this group of neurogenic transcription factors may contribute to the establishment of neuronal identity in the  
20 nervous system by acting on an overlapping but non-congruent set of target genes.

NeuroD proteins are transiently expressed in differentiating neurons during embryogenesis. NeuroD proteins are also detected in adult brain, in the granule layer of the hippocampus and the cerebellum. In addition, murine neuroD1 expression has been detected in the pancreas and gastrointestinal tissues of developing embryos and  
25 post-natal mice (see, e.g., Example 14). NeuroD proteins contain the basic helix-loop-helix (bHLH) domain structure that has been implicated in the binding of bHLH proteins to upstream recognition sequences and activation of downstream target genes. Based on homology with other bHLH proteins, the bHLH domain for murine neuroD1 is predicted to reside between amino acids 102 and 155 of SEQ ID NO:2, and between amino acids 101 and 157 of SEQ ID NO:4 for the amphibian neuroD1.  
30

NeuroD proteins are transcriptional activators that control transcription of downstream target genes including genes that among other activities cause neuronal progenitors to differentiate into mature neurons. In the neural stage of the mouse embryo (e10), murine neuroD1 is highly expressed in the neurogenic derivatives of  
35 neural crest cells, the cranial and dorsal root ganglia, and postmitotic cells in the

central nervous system (CNS). During mouse development, neuroD1 is expressed transiently and concomitant with neuronal differentiation in differentiating neurons in sensory organs such as in nasal epithelium and retina. In *Xenopus* embryos, ectopic expression of neuroD1 in non-neuronal cells induced formation of neurons. As  
5 discussed in more detail below, neuroD proteins are expressed in differentiating neurons and are capable of causing the conversion of non-neuronal cells into neurons. The present invention encompasses variants of neuroD genes that, for example, are modified in a manner that results in a neuroD protein capable of binding to its recognition site, but unable to activate downstream genes. The present invention also  
10 encompasses fragments of neuroD proteins that, for example, are capable of binding the natural neuroD partner, but that are incapable of activating downstream genes. NeuroD proteins encompass proteins retrieved from naturally occurring materials and closely related, functionally similar proteins retrieved by antisera specific to neuroD proteins, and recombinantly expressed proteins encoded by genetic materials (DNA,  
15 RNA, cDNA) retrieved on the basis of their similarity to the unique regions in the neuroD family of genes.

The present invention provides representative isolated and purified polynucleotide molecules encoding proteins of the neuroD family. Polynucleotide molecules encoding neuroD include those sequences resulting in minor genetic  
20 polymorphisms, differences between species, and those that contain amino acid substitutions, additions, and/or deletions. According to the present invention, polynucleotide molecules encoding neuroD proteins encompass those molecules that encode neuroD proteins or peptides that share identity with the sequences shown in SEQ ID NOS:2, 4, 9, 11, 13, 15, and 17.

25 In some instances, one may employ such changes in the sequence of a recombinant neuroD polynucleotide molecule to substantially decrease or even increase the biological activity of neuroD protein relative to the wild-type neuroD activity, depending on the intended use of the preparation. Such changes may also be directed towards endogenous neuroD polynucleotide sequences using, for example,  
30 gene therapy methods to alter the gene product. Such changes are envisioned with regard to neuroD1, neuroD2, neuroD3, or other members of the neuroD gene family.

The neuroD1 proteins of the present invention are capable of inducing the expression in a frog embryo of neuron-specific genes, such as N-CAM,  $\beta$ -tubulin, and Xen-1, neurofilament M (NF-M), Xen-2, tanabin-1, shaker-1, and frog HSCL. As  
35 described below in Example 10, neuroD1 activity may be detected when neuroD is

ectopically expressed in frog oocytes following, for example, injection of *Xenopus* neuroD1 RNA into one of the two cells in a two-cell stage *Xenopus* embryo, and monitoring expression of neuronal-specific genes in the injected as compared to uninjected side of the embryo by immunochemistry or *in situ* hybridization.

- 5 "Over-expression" means an increased level of a neuroD protein or of neuroD transcripts in a recombinant transformed host cell or in a tumor cell relative to the level of protein or transcripts in the untransformed host cell or in the normal cell from which the tumor is derived.

As noted above, the present invention provides isolated and purified  
10 polynucleotide molecules encoding various members of the neuroD family. The disclosed sequences may be used to identify and isolate additional neuroD polynucleotide molecules from suitable mammalian or non-mammalian host cells such as canine, ovine, bovine, caprine, lagomorph, or avian. In particular, the nucleotide sequences encoding the HLH region may be used to identify polynucleotide molecules  
15 encoding other proteins of the neuroD family. Complementary DNA molecules encoding neuroD family members may be obtained by constructing a cDNA library mRNA from, for example, fetal brain, newborn brain, and adult brain tissues. DNA molecules encoding neuroD family members may be isolated from such a library using the disclosed sequences to provide probes to be used in standard hybridization  
20 methods (e.g., Sambrook et al., *Molecular Cloning: A Laboratory Manual, Second Edition*, Cold Spring Harbor, NY, 1989, which is incorporated herein by reference), and Bothwell, Yancopoulos and Alt, *ibid.*) or by amplification of sequences using polymerase chain reaction (PCR) amplification (e.g., Loh et al., *Science* **243**:217-222, 1989; Frohman et al., *Proc. Natl. Acad. Sci. USA* **85**:8998-9002, 1988; Erlich (ed.),  
25 *PCR Technology: Principles and Applications for DNA Amplification*, Stockton Press, 1989; and Mullis et al., *PCR: The Polymerase Chain Reaction*, 1994, which are incorporated by reference herein in their entirety). In a similar manner, genomic DNA encoding neuroD proteins may be obtained using probes designed from the sequences disclosed herein. Suitable probes for use in identifying neuroD genes or  
30 transcripts may be obtained from neuroD-specific sequences that are highly conserved regions between mammalian and amphibian neuroD coding sequences. Nucleotide sequences, for example, from the region encoding the approximately 40 residues following the helix-2 domain are suitable for use in designing PCR primers. Alternatively, oligonucleotides containing specific DNA sequences from a human  
35 neuroD1, neuroD2, or neuroD3 coding region may be used within the described

methods to identify related human neuroD genomic and cDNA clones. Upstream regulatory regions of neuroD may be obtained using the same methods. Suitable PCR primers are between 7-50 nucleotides in length, more preferably between 15 and 25 nucleotides in length. Typically, probes must be at least 10 nucleotides in length to form stable hybrids. Alternatively, neuroD polynucleotide molecules may be isolated using standard hybridization techniques with probes of at least about 15 nucleotides in length and up to and including the full coding sequence. Southern analysis of mouse genomic DNA probed with the murine neuroD1 cDNA under stringent conditions showed the presence of only one gene, suggesting that under stringent conditions bHLH genes from other protein families will not be identified. Other members of the neuroD family can be identified using degenerate oligonucleotides based on the sequences disclosed herein for PCR amplification or by hybridization at moderate stringency using probes based on the disclosed sequences.

The regulatory regions of neuroD may be useful as tissue-specific promoters. Such regulatory regions may find use in, for example, gene therapy to drive the tissue-specific expression of heterologous genes in pancreatic, gastrointestinal, or neural cells, tissues or cell lines. As shown in Example 14, murine neuroD1 promoter sequences reside within the 1.4 kb 5' untranslated region. Regulatory sequences within this region are identified by comparison to other promoter sequences and/or deletion analysis of the region itself.

In other aspects of the invention, a DNA molecule coding a neuroD protein is inserted into a suitable expression vector, which is in turn used to transfect or transform a suitable host cell. Suitable expression vectors for use in carrying out the present invention include a promoter capable of directing the transcription of a polynucleotide molecule of interest in a host cell and may also include a transcription termination signal, these elements being operably linked in the vector. Representative expression vectors may include both plasmid and/or viral vector sequences. Suitable vectors include retroviral vectors, vaccinia viral vectors, CMV viral vectors, BLUESCRIPT vectors, baculovirus vectors, and the like. Promoters capable of directing the transcription of a cloned gene or cDNA may be inducible or constitutive promoters and include viral and cellular promoters. For expression in mammalian host cells, suitable viral promoters include the immediate early cytomegalovirus promoter (Boshart et al., *Cell* 41:521-530, 1985) and the SV40 promoter (Subramani et al., *Mol. Cell. Biol.* 1:854-864, 1981). Suitable cellular promoters for expression of proteins in mammalian host cells include the mouse metallothionein-1 promoter

(Palmiter et al., U.S. Patent No. 4,579,821), a mouse V<sub>k</sub> promoter (Bergman et al., *Proc. Natl. Acad. Sci. USA* 81:7041-7045, 1983; Grant et al. *Nucleic Acid. Res.* 15:5496, 1987), and tetracycline-responsive promoter (Gossen and Bujard, *Proc. Natl. Acad. Sci. USA* 89:5547-5551, 1992, and Pescini et al., *Biochem. Biophys. Res. Comm.* 202:1664-1667, 1994). Also contained in the expression vectors, typically, is a transcription termination signal located downstream of the coding sequence of interest. Suitable transcription termination signals include the early or late polyadenylation signals from SV40 (Kaufman and Sharp, *Mol. Cell. Biol.* 2:1304-1319, 1982), the polyadenylation signal from the Adenovirus 5 e1B region, and the human growth hormone gene terminator (DeNoto et al., *Nucleic Acid. Res.* 9:3719-3730, 1981). Mammalian cells, for example, may be transfected by a number of methods including calcium phosphate precipitation (Wigler et al., *Cell* 14:725, 1978; Corsaro and Pearson, *Somatic Cell Genetics* 7:603, 1981; Graham and Van der Eb, *Virology* 52:456, 1973), lipofection, microinjection, and electroporation (Neumann et al., *EMBO J.* 1:8410845, 1982). Mammalian cells can be transduced with viruses such as SV40, CMV, and the like. In the case of viral vectors, cloned DNA molecules may be introduced by infection of susceptible cells with viral particles. Retroviral vectors may be preferred for use in expressing neuroD proteins in mammalian cells particularly if the *neuroD* genes used for gene therapy (for review, see, Miller et al. *Methods in Enzymology* 217:581-599, 1994; which is incorporated herein by reference in its entirety). It may be preferable to use a selectable marker to identify cells that contain the cloned DNA. Selectable markers are generally introduced into the cells along with the cloned DNA molecules and include genes that confer resistance to drugs, such as neomycin, hygromycin, and methotrexate. Selectable markers may also complement auxotrophs in the host cell. Yet other selectable markers provide detectable signals, such as  $\beta$ -galactosidase to identify cells containing the cloned DNA molecules. Selectable markers may be amplifiable. Such amplifiable selectable markers may be used to amplify the number of sequences integrated into the host genome.

As would be evident to one of ordinary skill in the art, the polynucleotide molecules of the present invention may be expressed in *Saccharomyces cerevisiae*, filamentous fungi, and *E. coli*. Methods for expressing cloned genes in *Saccharomyces cerevisiae* are generally known in the art (see, "Gene Expression Technology," *Methods in Enzymology*, Vol. 185, Goeddel (ed.), Academic Press, San Diego, CA, 1990; and "Guide to Yeast Genetics and Molecular Biology," *Methods in*



*Enzymology*, Guthrie and Fink (eds.), Academic Press, San Diego, CA, 1991, which are incorporated herein by reference). Filamentous fungi may also be used to express the proteins of the present invention; for example, strains of the fungi *Aspergillus* (McKnight et al., U.S. Patent No. 4,935,349, which is incorporated herein by reference). Methods for expressing genes and cDNAs in cultured mammalian cells and in *E. coli* are discussed in detail in Sambrook et al., 1989. As will be evident to one skilled in the art, one can express the protein of the instant invention in other host cells such as avian, insect, and plant cells using regulatory sequences, vectors and methods well established in the literature.

NeuroD proteins produced according to the present invention may be purified using a number of established methods such as affinity chromatography using anti-neuroD antibodies coupled to a solid support. Fusion proteins of antigenic tag and neuroD can be purified using antibodies to the tag. Additional purification may be achieved using conventional purification means such as liquid chromatography, gradient centrifugation, and gel electrophoresis, among others. Methods of protein purification are known in the art (see generally, Scopes, R., *Protein Purification*, Springer-Verlag, NY, 1982, which is incorporated herein by reference) and may be applied to the purification of recombinant neuroD described herein.

The invention provides isolated and purified polynucleotide molecules encoding neuroD proteins that are capable of hybridizing under stringent conditions to an oligonucleotide of 15 or more contiguous nucleotides of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, and/or SEQ ID NO:16, and also including the polynucleotide molecules complementary to the coding strands. The subject isolated neuroD polynucleotide molecules preferably encode neuroD proteins that trigger differentiation in ectodermal cells, particularly neuroectodermal stem cells, and in more committed cells of that lineage, for example, epidermal precursor cells, pancreatic and gastrointestinal cells. Such neuroD expression products typically form heterodimeric bHLH protein complexes that bind in the 5'-regulatory regions of target genes and enhance or suppress transcription of the target gene.

In some instances, cancer cells may contain a non-functional neuroD protein or may contain no neuroD protein due to genetic mutation or somatic mutations such that these cells fail to differentiate. For cancers of this type, the cancer cells may be treated in a manner to cause the over-expression of wild-type neuroD protein to force differentiation of the cancer cells. Detection of overexpressed neuroD or other

neurogenic differentiation factors may serve to identify different types of brain tumors.

Antisense neuroD nucleotide sequences, that is, nucleotide sequences complementary to the non-transcribed strand of a neuroD gene, may be used to block expression of mutant neuroD expression in neuronal precursor cells to generate and harvest neuronal stem cells or, alternatively, to suppress inappropriately expressed neuroD in tumor cells. The use of antisense oligonucleotides and their applications have been reviewed in the literature (see, for example, Mol and Van der Krul, eds., *Antisense Nucleic Acids and Proteins Fundamentals and Applications*, New York, NY, 1992; which is incorporated by reference herein in its entirety). Suitable antisense oligonucleotides are at least 11 nucleotide in length and may include untranslated (upstream or intron) and associated coding sequences. Suitable target sequences for antisense oligonucleotides include intron-exon junctions (to prevent proper splicing), regions in which DNA/RNA hybrids will prevent transport of mRNA from the nucleus to the cytoplasm, initiation factor binding sites, ribosome binding sites, sites that interfere with ribosome progression, and 5' untranslated regions (promoter/enhancer) of the target gene. Antisense oligonucleotides may be prepared synthetically or by the insertion of a DNA molecule containing the target DNA sequence into a suitable expression vector such that the DNA molecule is inserted downstream of a promoter in a reverse orientation as compared to the gene itself. The expression vector may then be transduced, transformed or transfected into a suitable cell resulting in the expression of antisense oligonucleotides. Synthetic oligonucleotides may be introduced, e.g., by electroporation, calcium phosphate precipitation, liposomes, or microinjection. Synthetic antisense oligonucleotides may be stabilized, e.g., by using intercalating agents that are covalently attached to either or both ends of the oligonucleotide, or by being made nuclease resistant by modifications to the phosphodiester backbone by the introduction of phosphotriesters, phosphonates, phosphorothioates, phosphoroselenoates, phosphoramidates, phosphorodithioates, or by using alpha-anomers of the deoxyribonucleotides.

NeuroD proteins bind to 5' regulatory regions of neurogenic genes that are involved in neuroectodermal differentiation, including development of neural and endocrine tissues. As described in the Examples given below, murine neuroD1 has been detected in neuronal, pancreatic and gastrointestinal tissues in embryonic and adult mice suggesting that neuroD1 functions in the transcription regulation in these tissues. NeuroD proteins alter the expression of subject genes by, for example, down-

regulating or up-regulating transcription, or by inducing a change in transcription to an alternative open reading frame.

DNA sequences recognized by the various neuroD proteins may be determined using a number of methods known in the literature including immunoprecipitation (Biedenkapp et al., *Nature* 335:835-837, 1988; Kinzler and Vorgelstein, *Nuc. Acids Res.* 17:3645-3653, 1989; and Sompayrac and Danna, *Proc. Natl. Acad. Sci. USA* 87:3274-3278, 1990; which are incorporated by reference herein), protein affinity columns (Oliphant et al., *Mol. Cell. Biol.* 9:2944-2949, 1989; which is incorporated by reference herein), gel mobility shifts (Blackwell and Weintraub, *Science* 250:1104-1110, 1990; which is incorporated by reference herein), and Southwestern blots (Keller and Maniatis, *Nuc. Acids Res.* 17:4675-4680, 1991; which is incorporated by reference herein).

One embodiment of the present invention involves the construction of interspecies hybrid neuroD proteins and hybrid neuroD proteins containing at least one domain from two or more neuroD family members to facilitate structure-function analyses or to alter neuroD activity by increasing or decreasing the neuroD-mediated transcriptional activation of neurogenic genes relative to the wild-type neuroD(s). Hybrid proteins of the present invention may contain the replacement of one or more contiguous amino acids of the native neuroD protein with the analogous amino acid(s) of neuroD from another species or other protein of the neuroD family. Such interspecies or interfamilial hybrid proteins include hybrids having whole or partial domain replacements. Such hybrid proteins are obtained using recombinant DNA techniques. Briefly, DNA molecules encoding the hybrid neuroD proteins of interest are prepared using generally available methods such as PCR mutagenesis, site-directed mutagenesis, and/or restriction digestion and ligation. The hybrid DNA is then inserted into expression vectors and introduced into suitable host cells. The biological activity may be assessed essentially as described in the assays set forth in more detail in the Examples that follow.

The invention also provides synthetic peptides, recombinantly derived peptides, fusion proteins, and the like that include a portion of neuroD or the entire protein. The subject peptides have an amino acid sequence encoded by a nucleic acid which hybridizes under stringent conditions with an oligonucleotide of 15 or more contiguous nucleotides of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:8, SEQ ID NO:10, SEQ ID NO:12, SEQ ID NO:14, or SEQ ID NO:16. Representative amino acid sequences of the subject peptides are disclosed in SEQ ID NO:2, SEQ ID NO:4,

SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15, and SEQ ID NO:17. The subject peptides find a variety of uses, including preparation of specific antibodies and preparation of agonists and antagonists of neuroD activity.

As noted above, the invention provides antibodies that bind to neuroD proteins. The production of non-human antisera or monoclonal antibodies (e.g.,  
5 murine, lagormorph, porcine, equine) is well known and may be accomplished by, for example, immunizing an animal with neuroD protein or peptides. For the production of monoclonal antibodies, antibody producing cells are obtained from immunized animals, immortalized and screened, or screened first for the production of the  
10 antibody that binds to the neuroD protein or peptides and then immortalized. It may be desirable to transfer the antigen binding regions (e.g., F(ab')<sub>2</sub> or hypervariable regions) of non-human antibodies into the framework of a human antibody by recombinant DNA techniques to produce a substantially human molecule. Methods for producing such "humanized" molecules are generally well known and described in,  
15 for example, U.S. Patent No. 4,816,397; which is incorporated by reference herein in its entirety. Alternatively, a human monoclonal antibody or portions thereof may be identified by first screening a human B-cell cDNA library for DNA molecules that encode antibodies that specifically bind to the neuroD family member, e.g., according to the method generally set forth by Huse et al. (*Science* 246:1275-1281, 1989, which  
20 is incorporated by reference herein in its entirety). The DNA molecule may then be cloned and amplified to obtain sequences that encode the antibody (or binding domain) of the desired specificity.

The invention also provides methods for inducing the expression of genes, such as neurotransmitters or neuromodulatory factors, that are associated with  
25 neuronal phenotype in a cell that does not normally express those genes. For example, the modulation of gene expression by neuroD can be carried out in cells of the neuroectodermal lineage, glial cells, neural crest cells, and epidermal epithelial basal stem cells, and all types of both mesodermal and endodermal lineage cells. NeuroD expression may also be used as a means of inducing expression of genes  
30 associated with pancreatic and gastrointestinal phenotype, e.g., insulin or gastrointestinal-specific enzymes.

As illustrated in Example 10, the expression of *Xenopus* neuroD1 protein in stem cells causes redirection of epidermal cell differentiation and induces terminal differentiation into neurons, i.e., instead of epidermal cells. Epithelial basal stem cells  
35 (i.e., in skin and mucosal tissues) are one of the few continuously regenerating cell

types in an adult mammal. Introduction of the subject nucleotide sequences into an epithelial basal stem cell may be designed to achieve transient, constitutive, or regulated expression, and may be accomplished *in vitro* or *in vivo* using a suitable gene therapy vector delivery system (e.g., a retroviral vector), a microinjection technique (see, for example, Tam, *Basic Life Sciences* 37:187-194, 1986, which is  
5 incorporated by reference herein in its entirety), or a transfection method (e.g., naked or liposome encapsulated DNA or RNA; see, for example, *Trends in Genetics* 5:138, 1989; Chen and Okayama, *Biotechniques* 6:632-638, 1988; Mannino and Gould-Fogerite, *Biotechniques* 6:682-690, 1988; Kojima et al., *Biochem. Biophys. Res.*  
10 *Comm.* 207:8-12, 1995; which are incorporated by reference herein in their entirety).

Transformed host cells of the present invention are useful *in vitro* as convenient sources of neuronal and other growth factors for screening anti-cancer drugs capable of driving terminal differentiation in neural tumors, as sources of recombinantly expressed neuroD protein for use as an antigen in preparing  
15 monoclonal and polyclonal antibodies useful in diagnostic assays, and for screening for compounds capable of increasing or decreasing the activity of neuroD.

Transformed host cells of the present invention are also useful *in vivo* for transplantation at sites of traumatic neural injury where motor or sensory neural activity has been lost, e.g., for treating patients with hearing or vision loss due to  
20 optical or auditory nerve damage, patients with peripheral nerve damage and loss of motor or sensory neural activity, and patients with brain or spinal cord damage from traumatic injury or radiation injury. For example, donor cells from a patient such as epithelial basal stem cells are cultured *in vitro* and then transformed or transduced with a neuroD nucleotide sequence. The transformed cells are then returned to the  
25 patient by microinjection at the site of neural dysfunction. In addition, as neuroD appears capable of regulating expression of insulin, transformed host cells of the present invention may be useful for transplantation into patients with diabetes. For example, donor cells from a patient such as fibroblasts, pancreatic islet cells, or other pancreatic cells are harvested and transformed or transfected with a neuroD  
30 nucleotide sequence. The genetically engineered cells are then returned to the patient. In another embodiment, such engineered host cells may find use in the treatment of malabsorption syndromes or gastrointestinal dysmotility syndromes (Hirsh Prung's Disease).

Furthermore, the nucleotide sequences of the subject invention are useful for  
35 constructing cDNA and oligonucleotide probes for Northern or Southern blots, dot-

blots, or PCR assays for identifying and quantifying the level of expression of neuroD in a cell. In addition, birth defects and spontaneous abortions may result from expression of an abnormal neuroD protein; thus, screening neuroD expression may be useful in prenatal screening of mothers *in utero*.

5           The neuroD sequences of the subject invention are also useful for constructing recombinant cell lines, ova, and transgenic embryos and animals including dominant-negative and "knock-out" recombinant cell lines in which the transcription regulatory activity of neuroD protein is down-regulated or eliminated. Such cells may contain altered neuroD coding sequences that result in the expression of a defective neuroD  
10 protein that is not capable of enhancing, suppressing or activating transcription of its target gene(s). The subject cell lines and animals find uses in screening for candidate therapeutic agents capable of either substituting for neuroD or correcting the cellular defect caused by a defective neuroD. Alternatively, cell lines expressing wild-type neuroD proteins may be useful for correcting birth defects that result from defective  
15 neuroD expression.

          In addition, neuroD polynucleotide molecules may be joined to reporter genes, such as  $\beta$ -galactosidase or luciferase, and inserted into the genome of a suitable embryonic host cell such as a mouse embryonic stem cell by, for example, homologous recombination (for review, see Capecchi, *Trends in Genetics* 5:70-76,  
20 1989; which is incorporated by reference). Cells expressing neuroD may then be obtained by subjecting the differentiating embryonic cells to cell sorting, leading to the purification of a population neuroblasts that are useful for studying neuroblast sensitivity to growth factors or chemotherapeutic agents that may be used as a source of neuroblast-specific protein products or gene transcripts.

25           As illustrated in Example 14, neuroD1 "knock-out" mice had diabetes, as demonstrated by blood glucose levels 2 and 3 times that of wild-type mice, and they died within four days of birth, while heterozygous mutants exhibited wild-type blood glucose levels. These results suggest that neuroD1 "knock-out" mice may be useful for studying methods to rescue homozygous mutants and as hosts to test transplant  
30 tissue for treating diabetes. These results suggest further that *in vivo* correction of neuroD1 deficiencies may therapeutically benefit diabetes patients.

          The subject neuroD genes also are useful for constructing gene transfer vectors (e.g., retroviral vectors, and the like) wherein neuroD is inserted into the coding region of the vector under the control of a promoter. NeuroD gene therapy  
35 may be used to correct traumatic neural injury that has resulted in loss of motor or

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5 sensory neural function. For these therapies, gene transfer vectors may either be injected directly at the site of the traumatic injury, or the vectors may be used to construct transformed host cells that are then injected at the site of the traumatic injury. The results disclosed in Example 10 indicate that introduction of neuroD1 induces a non-neuronal cell to become a neuron, suggesting that transplantation and/or gene therapy with neuroD1 could be used to repair neural defects resulting from traumatic injury. NeuroD1 also may be useful for treating neurological disorders such as Alzheimer's disease, Huntington's disease, and Parkinson's disease, in which a population of neurons have been damaged. For such therapies, recombinant neuroD1 sequences may be introduced into existing neurons, or endogenous neuroD1 expression is induced in existing neurons *in vivo*. Alternatively, neuroD1 expression is induced in non-neuronal cells (e.g., glial cells in the brain or basal epithelial cells) to induce expression of genes that confer a complete or partial neuronal phenotype that ameliorates aspects of the disease. As an example, Parkinson's disease is caused, at least in part, by the death of neurons that supply the neurotransmitter dopamine to the basal ganglia. Increasing the levels of neurotransmitter ameliorates the symptoms of Parkinson's disease. Expression of neuroD1 in basal ganglia neurons or glial cells may induce aspects of a neuronal phenotype such that the neurotransmitter dopamine is produced directly in these cells. Alternatively, donor cells expressing a neuroD gene could be transplanted into the affected region. Also, neuroD1 can be expressed in non-pancreatic cells to induce expression of genes that confer a complete or partial pancreatic phenotype that ameliorates aspects of diabetes. Within yet another embodiment, neuroD1 is expressed in pancreatic islet cells to induce expression of genes that induce the expression of insulin.

25 The subject neuroD genes also are useful for the preparation of transplantable recombinant neuronal precursor cell populations from embryonic ectodermal cells, non-neural basal stem cells, and the like. The isolated polynucleotide molecules encoding neuroD proteins of the present invention permit the establishment of primary (or continuous) cultures of proliferating embryonic neuronal stem cells under conditions mimicking those that are active in development and cancer. The resultant cell lines find uses: i) as sources of novel neural growth factors, ii) in screening assays for anti-cancer compounds, and iii) in assays for identifying novel neuronal growth factors. For example, a high level of expression of neuroD was observed in the embryonic optic tectum, indicating that neuroD1 protein may regulate expression of

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factors trophic for growing retinal cells. Such cells may be useful sources of growth factors, and may be useful in screening assays for candidate therapeutic compounds.

The cell lines and transcription regulatory factors disclosed herein offer the unique advantage that since they are active very early in embryonic differentiation they represent potential switches, e.g., ON→OFF or OFF→ON, controlling subsequent cell fate. If the switch can be shown to be reversible (i.e., ON↔OFF), the neuroD genes and proteins disclosed herein provide exciting opportunities for restoring lost neural and/or endocrine functions in a subject.

The following examples are offered by way of illustration and not by way of limitation.

### EXAMPLE 1

#### Construction of the embryonic stem cell "179" cDNA library.

A continuous murine embryonic stem cell line (i.e., the ES cell line) having mutant E2A (the putative binding partner of myoD) was used as a cell source to develop a panel of embryonic stem cell tumors. Recombinant ES stem cells were constructed (i.e., using homologous recombination) wherein both alleles of the putative myoD binding partner E2A were replaced with drug-selectable marker genes. ES cells do not make functional E12 or E47 proteins, both of which are E2A gene products. ES cells form subcutaneous tumors in congenic mice (i.e., strain 129J) that appear to contain representatives of many different embryonal cell types as judged histologically and through the use of RT-PCR gene expression assays. Individual embryonic stem cell tumors were induced in male 129J strain mice by subcutaneous injection of  $1 \times 10^7$  cells/site. Three weeks later each tumor was harvested and used to prepare an individual sample of RNAs. Following random priming and second strand synthesis the ds-cDNAs were selected based on their size on 0.7% agarose gels and those cDNAs in the range of 400-800 bp were ligated to either Bam HI or Bgl II linkers. (Linkers were used to minimize the possibility that an internal Bam HI site in a cDNA might inadvertently be cut during cloning, leading to an abnormally sized or out-of-frame expression product.) The resultant individual stem cell tumor DNAs were individually ligated into the Bam HI cloning site in the "fl-VP16" 2 $\mu$  yeast expression vector. This expression vector, fl-VP16, contains the VP16 activation domain of Herpes simplex virus (HSV) located between Hind III (HIII) and Eco RI (RI) sites and under the control of the *Saccharomyces cerevisiae* alcohol dehydrogenase promoter; with *LEU2* and Ampicillin-resistance selectable markers. Insertion of a DNA molecule of interest into the Hind III site of the fl-VP16 vector



(i.e., 5' to the VP16 nucleotide sequence), or into a Bam HI site (i.e., 3' to the VP16 sequence but 5' to the Eco RI site), results in expression of a VP16 fusion protein having the protein of interest joined in-frame with VP16. The resultant cDNA library was termed the "179-library".

5

## EXAMPLE 2

### Identification and cDNA cloning of mouse neuroD1.

A two-hybrid yeast screening assay was used essentially as described by Fields and Song (*Nature* 340:245, 1989) and modified as described herein was used to screen the 179-library described in Example 1. Yeast two-hybrid screens are reviewed as disclosed in Fields and Sternglanz (*Trends in Genetics* 10:286-292, 1994). The library was screened for cDNAs that interacted with LexA-Da, a fusion protein between the *Drosophila* Da (Daughterless) bHLH domain and the prokaryotic LexA-DNA binding domain. The *S. cerevisiae* strain L40 contained multimerized LexA binding sites cloned upstream of two reporter genes, namely, the *HIS3* gene, and the  $\beta$ -galactosidase gene, each of which was integrated into the L40 genome. The *S. cerevisiae* strain L40 containing a plasmid encoding the LexA-Da fusion protein was transformed with CsCl gradient-purified fl-VP16-179-cDNA library. Transformants were maintained on medium selecting both plasmids (the LexA-Da plasmid and the cDNA library plasmid) for 16 hours before being subjected to histidine selection on plates lacking histidine, leucine, tryptophan, uracil, and lysine. Clones that were  $HIS^+$  were subsequently assayed for the expression of *LacZ*. To eliminate possible non-specific cloning artifacts, plasmids from  $HIS^+/LacZ^+$  were isolated and transformed into *S. cerevisiae* strain L40 containing a plasmid encoding a LexA-Lamin fusion. Clones that scored positive in the interaction with lamin were discarded. Approximately 400 cDNA clones, which represented 60 different transcripts, were identified as positive in these assays. Twenty-five percent of the original clones were subsequently shown to be known bHLH genes on the basis of their reactivity with specific cDNA probes. One cDNA clone encoding a VP16-fusion protein that interacted with Da but not lamin was identified as unique by sequence analysis. This clone, initially termed *tango*, is now referred to as neuroD1.

30

The unique cDNA identified above, VP16-neuroD, contained an approximately 450 bp insert that spanned the bHLH region. Sequence analysis showed that the clone contained an insert encoding a complete bHLH amino acid sequence motif that was unique and previously unreported. Further analysis suggested that while the cDNA contained conserved residues common to all members

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of the bHLH protein family, several residues were unique and made it distinct from previously identified bHLH proteins. The DNA cloned in VP16-neuroD is referred to as "neuroD1." The neuroD1 cDNA insert was subcloned as a Bam HI-Not I insert into Bam HI-Not I linearized pBluescript SK<sup>+</sup>. The resulting plasmid was designated pSK+1-83.

The neuroD1 insert contained in the VP16-neuroD plasmid was used to reprobe a mouse cDNA library prepared from mouse embryos at developmental stage e10.5. Candidate clones were isolated and sequenced essentially as described above. Several clones were isolated. One clone, designated pKS<sup>+</sup> m7a RX, was deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852 USA, on May 6, 1994, under accession number 75768. Plasmid pKS<sup>+</sup> m7a RX contains 1646 bp of murine neuroD1 cDNA as an EcoRI-XhoI insert. The amino acid sequence encoded by the insert begins at amino acid residue +73 and extends to the carboxy-terminus of the neuroD1 protein. The plasmid contains about 855 bp of neuroD1 coding sequence (encoding amino acids 73-536).

None of the mouse cDNAs contained the complete 5' coding sequence. To obtain the 5' neuroD1 coding sequence, a mouse strain 129/Sv genomic DNA library was screened with the VP16-neuroD plasmid insert (450 bp). Genomic clones were isolated and sequenced and the sequences were aligned with the cDNA sequences. Alignment of the sequence and comparison of the genomic 5' coding sequences with the *Xenopus* neuroD1 clone (Example 8) confirmed the 5' neuroD1 coding sequence. The complete neuroD1 coding sequence and deduced amino acid sequence are shown in SEQ ID NOS:1 and 2.

### EXAMPLE 3

#### NeuroD/neuroD

bHLH proteins share common structural similarities that include a basic region that binds DNA and an HLH region involved in protein-protein interactions required for the formation of homodimers and heterodimeric complexes. A comparison of the amino acid sequence of the basic region of murine neuroD1 (amino acids 102 to 113 of SEQ ID NO:2) with basic regions of other bHLH proteins revealed that murine neuroD1 contained all of the conserved residues characteristic among this family of proteins. However, in addition, neuroD1 contained several unique residues. These unique amino acid residues were not found in any other known HLH, making neuroD1 a distinctive new member of the bHLH family. The NARERNR basic region motif in neuroD1 (amino acids 107-113 of SEQ ID NO:2) is also found in the

*Drosophila* AS-C protein, a protein thought to be involved in neurogenesis. Similar, but not identical, NARERRR and NERERNR motifs (SEQ ID NOS:5 and 6, respectively) have been found in the *Drosophila* Atonal and MASH (mammalian achaete-scute homolog) proteins, respectively, which are also thought to be involved in neurogenesis. The NARER motif (SEQ ID NO:7) of neuroD1 is shared by other bHLH proteins, and the *Drosophila* Daughterless (Da) and Mammalian E proteins. The basic region of bHLH proteins is important for DNA binding site recognition, and there is homology between neuroD1 and other neuro-proteins in this functional region. Within the important dimer-determining HLH region of neuroD1, a low level of homology was recorded with mouse twist protein (i.e., 51% homology) and with MASH (i.e., 46% homology). NeuroD1 contains several regions of unique peptide sequence within the bHLH domain including the junction sequence (MHG).

#### EXAMPLE 4

##### Tissue expression patterns of neuroD1, neuroD2, and neuroD3

NeuroD1 expression was analyzed during embryonic development of mouse embryos using *in situ* hybridization. The probe used was an antisense neuroD1 single-stranded riboprobe labeled with digoxigenin (Boehringer Mannheim). Briefly, a riboprobe was prepared from plasmid pSK+1-83 using T7 polymerase and digoxigenin-11-UTP for labeling. The hybridized probe was detected using anti-digoxigenin antibody conjugated with alkaline phosphatase. Color development was carried out according to the manufacturer's instructions. Stages of development are commonly expressed as days following copulation and where formation of the vaginal plug is e0.5. The results recorded in the *in situ* hybridization studies were as follows:

In the e9.5 mouse embryo, neuroD1 expression was observed in the developing trigeminal ganglia.

In the e10.5 mouse embryo, a distinctive pattern of neuroD1 expression was observed in all the cranial ganglia (i.e., V-XI) and in dorsal root ganglia (DRG) in the trunk region of the embryo. At this time, neuroD1 expression was also observed in the central nervous system in post-mitotic cells in the brain and spinal cord that were undergoing neuronal differentiation. In the spinal cord, the ventral portion of the cord from which the motor neurons arise and differentiate was observed to express neuroD1 at high levels; and expression in the posterior-ventral spinal cord was higher when compared to more mature anterior-ventral spinal cord.

In the e11.5 mouse embryo, the ganglionic expression pattern of neuroD1 observed in e10.5 persisted. Expression in the spinal cord was increased over the

level of expression observed in e10.5 embryos, which was consistent with the presence of more differentiating neurons at this stage. At this stage neuroD1 expression was also observed in other sensory organs in which neuronal differentiation occurs, for example, in the nasal epithelium, otic vesicle, and retina of the eye. In both of these organs neuroD1 expression was observed in the region containing differentiating neurons.

In the e14.5 mouse embryo, expression of neuroD1 was observed in cranial ganglia and DRG, but expression of neuroD1 persisted in the neuronal regions of developing sensory organs and the central nervous system (CNS). Thus, neuroD1 expression was observed to be transient during neuronal development.

In summary, expression of neuroD1 in the neurula stage of the embryo (e10), in the neurogenic derivatives of neural crest cells, the cranial and dorsal root ganglia, and post mitotic cells in the CNS suggests an important possible link between expression and generation of sensory and motor nerves. Expression occurring later in embryonic development in differentiating neurons in the CNS and in sensory organs (i.e., nasal epithelium and retina) also supports a role in development of the CNS and sensory nervous tissue. Since neuroD1 expression was transient, the results suggest that neuroD1 expression is operative as a switch controlling formation of sensory nervous tissue. It is noteworthy that in these studies neuroD1 expression was not observed in embryonic sympathetic and enteric ganglia (also derived from migrating neural crest cells). Overall, the results indicate that neuroD1 plays an important role in neuronal differentiation.

In addition to the *in situ* studies described above, Northern blot analysis was done to determine in what tissues of the mouse neuroD1, neuroD2, and neuroD3 were expressed. Total RNA was isolated from whole mouse embryos and adult mouse tissues. RNA isolation was performed using RNazol B according to the protocol provided (Cinna/Biotex CS-105B). RNA was size fractionated on 1.5% agarose gels and transferred to Hybond-N membranes. Hybridization was carried out in 7% SDS, 0.25 M Na<sub>2</sub>PO<sub>4</sub>, 10mg/ml BSA, 1 mM EDTA at 65°C for at least 5 hours and then washed in 0.1X SSC and 0.1% SDS at 55°C-60°C. Probes for analyzing mouse mRNA were prepared from fragments representing the divergent carboxy-terminal regions 3-prime of the bHLH domain to avoid cross-hybridization between genes. Probe for neuroD1 was made from a 350 base pair PstI fragment from the mouse neuroD1 cDNA (Lee et al., 1995) that encompasses the region coding for amino acids 187-304; probe for neuroD2 was made from a 635 base pair

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PstI fragment from the mouse neuroD2 cDNA that encompasses the region from amino acid 210 through to the 3-prime non-translated region; and probe for neuroD3 was made from a 400 base pair ApaI-BamHI fragment from the neuroD3 genomic region that is 3-prime to the region coding the bHLH domain.

5       After labeling with <sup>32</sup>P, the above-described fragments were used to probe Northern blots containing RNAs prepared from various tissues of newborn and adult mice. Both neuroD1 and neuroD2 were detected in the brain of both newborn and adult mice, whereas, neuroD3 transcripts were not detected in any of the tissues tested. RNA extracted from dissected regions of the adult mouse nervous system  
10       demonstrated that neuroD1 was more abundant in the cerebellum than the cortex, whereas neuroD2 was expressed at relatively equivalent levels in both cerebellum and cortex.

To determine when during mouse embryonic development neuroD2 and neuroD3 were expressed in comparison to neuroD1, RNA was prepared from whole  
15       embryos at various developmental stages. In accord with previous reports (Lee et al., 1995), neuroD1 mRNA was first detected at low levels at embryonic day 9.5 and at increasing levels through embryonic day 12.5, the latest embryonic stage tested. NeuroD2 mRNA was first detected at embryonic day 11 and also increased in abundance through embryonic day 12.5. Although we did not detect neuroD3 in the  
20       adult tissues, the embryonic expression pattern showed a transient expression between embryonic day 10 and 12 and then declined to undetectable levels by embryonic day 16. Collectively, these data demonstrate that neuroD3 is expressed transiently during embryogenesis, similar to the expression pattern of *MATH1* (Akazawa et al., 1995), and that the temporal expression of neuroD1 and neuroD2 partly overlap with  
25       neuroD3, but that their expression persists in the adult nervous system.

#### EXAMPLE 5

##### NeuroD1 is expressed in neural and brain tumor cells:

##### murine probes identify human neuroD1.

Given the expression pattern in mouse embryo (Example 4), Northern blots of  
30       tumor cell line mRNAs were examined using murine neuroD1 cDNA (Example 2) as a molecular probe. As a first step, cell lines that have the potential for developing into neurons were screened. The D283 human medulloblastoma cell line, which expressed many neuronal markers, expressed high levels of neuroD1 by Northern blot analysis. NeuroD1 was also transcribed at various levels by different human neuroblastoma cell  
35       lines and in certain rhabdomyosarcoma lines that are capable of converting to neurons.

**EXAMPLE 6****Recombinant cells expressing NeuroD1.**

Recombinant murine 3T3 fibroblast cells expressing either a myc-tagged murine neuroD1 protein or myc-tagged *Xenopus* neuroD1 protein were made. The recombinant cells were used as a test system for identifying antibody to neuroD described below.

*Xenopus* neuroD1 protein was tagged with the antigenic marker Myc to allow the determination of the specificity of anti-neuroD1 antibodies to be determined. Plasmid CS2+MT was used to produce the Myc fusion protein. The CS2+MT vector (Turner and Weintraub, *ibid.*) contains the simian cytomegalovirus IE94 enhancer/promoter (and an SP6 promoter in the 5' untranslated region of the IE94-driven transcript to allow *in vitro* RNA synthesis) operatively linked to a DNA sequence encoding six copies of the Myc epitope tag (Roth et al, *J. Cell Biol.* 115:587-596, 1991; which is incorporated herein in its entirety), a polylinker for insertion of coding sequences, and an SV40 late polyadenylation site. CS2+MT was digested with Xho I to linearize the plasmid at the polylinker site downstream of the DNA sequence encoding the Myc tag. The linearized plasmid was blunt-ended using Klenow and dNTPs. A full length *Xenopus neuroD1* cDNA clone was digested with Xho I and Eae I and blunt-ended using Klenow and dNTPs, and the 1.245 kb fragment of the *Xenopus neuroD1* cDNA was isolated. The neuroD1 fragment and the linearized vector were ligated to form plasmid CS2+MT x1-83.

CS2+MT was digested with Eco RI to linearize the plasmid at the polylinker site downstream of the DNA sequence encoding the Myc tag. The linearized plasmid was blunt-ended using Klenow and dNTPs and digested with Xho I to obtain a linearized plasmid having an Xho I adhesive end and a blunt end. Plasmid pKS+m7a containing a partial murine neuroD1 cDNA was digested with Xho I, and the neuroD1 containing fragment was blunt-ended and digested with Xba I to obtain the approximately 1.6 kb fragment of the murine neuroD1 cDNA. The neuroD1 fragment and the linearized vector were ligated to form plasmid CS2+MT M1-83(m7a).

Plasmids CS2+MT x1-83 and CS2+MT M1-83(m7a) were each transformed into murine 3T3 fibroblast cells and used as a test system for identifying antibody against neuroD1 (Example 7).

### EXAMPLE 7

#### Antibodies to NeuroD1.

A recombinant fusion protein of maltose binding protein (MBP) and amino acid residues 70-355 of murine neuroD1 was used as an antigen to evoke antibodies in rabbits. Specificity of the resultant antisera was confirmed by immunostaining of the recombinant 3T3 cells described above. Double-immunostaining of the recombinant cells was observed with monoclonal antibodies to Myc (i.e., the control antigenic tag on the transfected DNA) and with rabbit anti-murine neuroD1 in combination with anti-rabbit IgG. The specificity of the resultant anti-murine neuroD1 sera was investigated further by preparing mouse 3T3 fibroblasts cells transfected with different portions of neuroD1 DNA. Specificity seemed to map to the glutamic acid-rich domain (i.e., amino acids 66-73 of SEQ ID NO:2). The anti-murine antisera did not react with cells transfected with the myc-tagged *Xenopus* neuroD1. In a similar manner, *Xenopus* neuroD1 was used to generate rabbit anti-neuroD antisera. The antisera was *Xenopus*-specific and did not cross react with cells transfected with Myc-tagged murine neuroD1.

### EXAMPLE 8

#### NeuroD1 is a highly evolutionarily conserved protein: sequence of *Xenopus* neuroD1.

Approximately one million clones from a stage 17 *Xenopus* head cDNA library made by Kintner and Melton (*Development* 99:311, 1987) were screened with the mouse cDNA insert as a probe at low stringency. The hybridization was performed with 50% formamide/4 X SSC at 33°C and washed with 2 X SSC/0.1% SDS at 40°C.

Positive clones were identified and sequenced. Analysis of the *Xenopus* neuroD1 cDNA sequence (SEQ ID NO:3) revealed that neuroD1 is a highly conserved protein between frog and mouse. The deduced amino acid sequences of frog and mouse (SEQ ID NOS:2 and 4) show 96% identity in the bHLH domain (50 of 52 amino acids are identical) and 80% identity in the region that is carboxy-terminal to the bHLH domain (159 of 198 amino acids are identical). The domain structures of murine and *Xenopus* neuroD1 are highly homologous with an "acidic" N-terminal domain (i.e., glutamic or aspartic acid rich); a basic region; helix 1, loop, helix 2; and a proline rich C-terminal region. Although the amino terminal regions of murine and *Xenopus* neuroD1 differ in amino acid sequence, both retain a glutamic or aspartic acid rich "acidic domain" (amino acids 102 to 113 of SEQ ID NO:2 and amino acids 56 to 79 of SEQ ID NO:4). It is highly likely that the acidic domain

constitutes an "activation" domain for the neuroD1 protein, in a manner analogous to the activation mechanisms currently understood for other known transcription regulatory factors.

#### EXAMPLE 9

##### 5                    Neuronal expression of *Xenopus* neuroD1.

The expression pattern of neuroD1 in whole mount *Xenopus* embryos was determined using *in situ* hybridization with a single stranded digoxigenin-labeled *Xenopus* neuroD1 antisense cDNA riboprobe. Embryos were examined at several different stages.

10                Consistent with the mouse expression pattern, by late stage, all cranial ganglia showed very strong staining patterns. In *Xenopus*, as in other vertebrate organisms, neural crest cells give rise to skeletal components of the head, all ganglia of the peripheral nervous system, and pigment cells. Among these derivatives, the cranial sensory ganglia, which are of mixed crest and placode origin, represent the only group  
15                of cells that express neuroD1. High levels of neuroD1 expression in the eye were also observed, correlating with active neuronal differentiation in the retina at this stage. Expression is observed in the developing olfactory placodes and otic vesicles, as was seen in mice. The pineal gland also expressed neuroD1. All of this expression was transient, suggesting that neuroD1 functions during the differentiation process but is  
20                not required for maintenance of these differentiated cell types.

As early as stage 14 (i.e., the mid-neurula stage) neuroD1 expression was observed in the cranial neural crest region where trigeminal ganglia differentiate. Primary mechanosensory neurons in the spinal cord, also referred to as Rohon-Beard cells and primary motor neurons, showed neuroD1 expression at this stage.

25                By stage 24, all of the developing cranial ganglia, trigeminal, facio-acoustic, glosso-pharyngeal, and vagal nervous tissues showed a high level of neuroD1 expression. High levels of expression of neuroD1 were also observed in the eye at this stage. (Note that in *Xenopus* neuronal differentiation in the retina occurs at a much earlier stage than in mice, and neuroD1 expression was correspondingly earlier  
30                and stronger in this animal model.)

In summary, in *Xenopus* as in mouse, neuroD1 expression was correlated with sites of neuronal differentiation. The remarkable evolutionary conservation of the pattern of neuroD1 expression in differentiating neurons supports the notion that neuroD1 has been evolutionarily conserved both structurally and functionally in these



distant classes, which underscores the critical role performed by this protein in embryonic development.

#### EXAMPLE 10

##### Expression of neuroD1 and neuroD2 converts non-neuronal cells into neurons.

5 To further analyze the biological functions of neuroD1, a gain-of-function assay was conducted. In this assay, RNA was microinjected into one of the two cells in a 2-cell stage *Xenopus* embryo, and the effects on later development of neuronal phenotype were evaluated. For these experiments *myc*-tagged *Xenopus* neuroD1 transcripts were synthesized *in vitro* using SP6 RNA polymerase. The *myc*-tagged-  
10 neuroD1 transcripts were microinjected into one of the two cells in a *Xenopus* 2-cell embryo, and the other cell of the embryo served as an internal control.

Synthesis of capped RNA for the *Xenopus laevis* injections was done essentially as described (Kreig, P. A. and D. A. Melton., *Meth. Enzymol.* 155:397-415, 1987) using the SP6 transcription of the pCS2-hND2, pCS2-hND1, pCS2-mND2, and pCS2MT-mND2. The capped RNA was phenol/chloroform extracted followed by separation of unincorporated nucleotides using a G-50 spin column. Approximately 350 pg of capped RNA was injected into one cell of 2-cell stage albino *Xenopus laevis* embryo in a volume of approximately 5 nl, as described previously (Turner and Weintraub, 1994). Embryos were allowed to develop in 0.1X modified  
20 Barth's saline (MBS) and staged according to Nieuwkoop and Faber (Nieuwkoop, P.D. and J. Faber, "Normal Table of *Xenopus laevis*," North-Holland Publishing Co., Amsterdam, Holland, 1967). Embryos were fixed in MEMFA for 2 hours at room temperature and stored in methanol. Embryos were hydrated through a graded series of methanol/PBS solutions and prepared for immunohistochemistry as described  
25 (Turner and Weintraub, 1994). The embryos were stained with an anti-NCAM antibody (Balak et al. *Develop. Biol.* 119:540-550, 1987) diluted 1:500 (gift of Urs Rutishauser) followed by a goat anti-rabbit alkaline phosphatase conjugated secondary antibody, or stained with the monoclonal anti-myc tag 9e10 antibody. Presence of the antibody was visualized by NBT/BCIP color reaction according to  
30 protocol provided (Gibco).

Antibodies to *Xenopus* N-CAM, a neural adhesion molecule, anti-Myc (to detect the exogenous protein tag), and immunostaining techniques were used to evaluate phenotypic expression of the neuronal marker (and control) gene during the subsequent developmental stages of the microinjected embryos. Remarkably, an  
35 evaluation of over 130 embryos that were injected with neuroD1 RNA showed a

striking increase in ectopic expression of N-CAM on the microinjected side of the embryo (i.e., Myc+), as judged by increased immunostaining. The increased staining was observed in the region from which neural crest cells normally migrate. It is considered likely that ectopic expression (or over-expression) of neuroD1 caused  
5 neural crest stem cells to follow a neurogenic cell fate. Outside the neural tube, the ectopic immunostaining was observed in the facio-cranial region and epidermal layer, and in some cases the stained cells were in the ventral region of the embryo far from the neural tube. The immunostained cells not only expressed N-CAM ectopically, but displayed a morphological phenotype of neuronal cells. At high magnification, the N-  
10 CAM expressing cells exhibited typical neuronal processes reminiscent of axonal processes.

To confirm that the ectopic N-CAM expression resulted from a direct effect on the presumptive epidermal cells and not from aberrant neural cell migration into the lateral and ventral epidermis, neuroD1 RNA was injected into the top tier of 32-  
15 cell stage embryos, in order to target the injection into cells destined to become epidermis. N-CAM staining was observed in the lateral and ventral epidermis without any noticeable effect on the endogenous nervous system, indicating that the staining of N-CAM in the epidermis represents the conversion of epidermal cell fate into neuronal cell fate.

Ectopic generation of neurons by neuroD1 was confirmed with other neural specific markers, such as neural-specific class II  $\beta$ -tubulin (Richter et al., *Proc. Natl. Acad. Sci. USA* 85:8066, 1988), acetylated  $\alpha$ -tubulin (Piperno and Fuller, *J. Cell. Biol.* 101:2085, 1985), tanabin (Hemmati-Brinvanlou et al., *Neuron* 9:417, 1992), neurofilament(NF)-M (Szaro et al., *J. Comp. Neurol.* 273:344, 1988), and Xen-1,2  
25 (Ruiz i Altaba, *Development* 115:67, 1992). The embryos were subjected to immunochemistry as described by Turner and Weintraub (*Genes Dev.* 8:1434, 1994, which is incorporated by reference herein) using primary antibodies detected with alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit antibodies diluted to 1:2000 (Boehringer-Mannheim). Anti-acetylated alpha-tubulin was diluted 1:2000.  
30 Anti-Xen-1 was diluted 1:1. Anti-NF-M was diluted 1:2000. Embryos stained for NF-M were fixed in Dent's fixative (20% dimethylsulfoxide/80% methanol) and cleared in 2:1 benzyl benzoate/benzyl alcohol as described by Dent et al. (*Development* 105:61, 1989, which is incorporated by reference herein). *In situ* hybridization of embryos was carried out essentially as described by Harland (in  
35 *Methods in Cell Biology*, B.K. Kay, H.J. Pend, eds., Academic Press, New York,

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NY, Vol. 36, pp. 675-685, 1991, which is incorporated by reference herein) as modified by Turner and Weintraub (ibid.). *In situ* hybridization with  $\beta$ -tubulin without RNase treatment can also detect tubulin expression in the ciliated epidermal cells. All of these markers displayed ectopic staining on the neuroD1 RNA injected side. Injection of neuroD1 mRNA into vegetal cells led to no ectopic expression of neural markers except in one embryo that showed internal N-CAM staining in the trunk region, suggesting the absence of cofactors or the presence of inhibitors in vegetal cells. However, the one embryo that showed ectopic neurons in the internal organ tissue suggests that it may be possible to convert non-ectodermal lineage cells into neurons under certain conditions.

The embryos were also stained with markers that detect Rohon-Beard cells (cells in which neuroD1 is normally expressed). Immunostaining using the method described above for Rohon-Beard cell-specific markers such as HNK-1 (Nordlander, *Dev. Brain Res.* 50:147, 1989, which is incorporated by reference herein) at a dilution of 1:1, Islet-1 (Ericson et al., *Science* 256:1555, 1992 and Korzh et al., *Development* 118:417, 1993) at a dilution of 1:500, and *in situ* hybridization as described above with shaker-1 (Ribera et al., *J. Neurosci.* 13:4988, 1993) showed more cells staining on the injected side of the embryos.

The combined results support the notion that ectopic expression of neuroD1 induced differentiation of neuronal cells from cells that, without neuroD1 microinjection, would have given rise to non-neuronal cells. In summary, these experiments support the notion that ectopic neuroD1 expression can be used to convert a non-neuronal cell (i.e., uncommitted neural crest cells and epidermal epithelial basal stem cells) into a neuron. These findings offer for the first time the potential for gene therapy to induce neuron formation in injured neural tissues.

Interesting morphological abnormalities were observed in the microinjected embryos. In many cases the eye on the microinjected side of the embryo failed to develop. In other embryos, the spinal cord on the microinjected side of the embryo failed to develop properly, and the tissues were strongly immunopositive when stained with anti-N-CAM. In addition, at the mid-neurula stage many microinjected embryos exhibited an increase in cell mass in the cranial region of the embryo from which (in a normal embryo) the neural crest cells and their derivatives (i.e., cranial ganglionic cells) would migrate. The observed cranial bulge exhibited strong immunostaining with antibodies specific for N-CAM. These results were interpreted to mean that morphological changes in the eye, neural crest, and spinal cord resulted from

premature neural differentiation which altered the migration of neural and neural crest precursor cells.

NeuroD1-injected embryos were also assayed for alteration in the expression of Xtwist, the *Xenopus* homolog of *Drosophila* twist, to determine whether neuroD1 converted non-neuronal components of neural crest cells into the neural lineage. In wild-type embryos, Xtwist is strongly expressed in the non-neuronal population cephalic neural crest cells that give rise to the connective tissue and skeleton of the head. NeuroD1-injected embryos were completely missing Xtwist expression in the migrating cranial neural crest cells on the injected side. The failure to generate sufficient cranial mesenchymal neural crest precursors in neuroD1-injected embryos was also observed morphologically, since many of the injected embryos exhibited poor branchial arch development in the head. Furthermore, the increased mass of cells in the cephalic region stained very strongly for N-CAM,  $\beta$ -tubulin, and Xen-1, indicating that these cells were neural in character.

The converse experiment in which frog embryos were injected with Xtwist mRNA showed that ectopic expression of Xtwist significantly decreased neuroD1 expression on the injected side. Thus, two members of the bHLH family, neuroD1 and Xtwist, may compete for defining the identity of different cell types derived from the neural crest. In the neuroD1-injected embryos, exogenous neuroD1 may induce premigratory neural crest to differentiate into neurons *in situ*, and consequently they fail to migrate to their normal positions.

The effect of introduction of exogenous neuroD1 on the fate of cells that normally express neuroD1, such as cranial ganglia, eye, otic vesicle, olfactory organs, and primary neurons, and on other CNS cells that normally do not express neuroD1, was determined by staining for differentiation markers. When the cranial region of the embryo was severely affected by ectopic neuroD1, the injected side of the embryos displayed either small or no eyes in addition to poorly organized brains, otic vesicles, and olfactory organs. Moreover, as the embryos grew, the spinal cord showed retarded growth, remaining thinner and shorter on the neuroD1-injected side.

N-CAM staining in the normal embryo at early stages was not uniform throughout the entire neural plate, but rather was more prominent in the medial region of the neural plate. Injected embryos analyzed for N-CAM expression showed that the neural plate on the injected side of the early stage embryos was stained more intensely and more laterally. The increase in N-CAM staining was not associated with any lateral expansion of the neural plate as assayed by visual inspection and staining

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with the epidermal marker EpA. This was in contrast to what has been observed with XASH-3 injection that causes neural plate expansion. These observations suggest that the first effects of neuroD1 are to cause neuronal precursors in the neural plate to differentiate prematurely.

5 To determine whether neuroD1 caused neuronal precursors to differentiate prematurely, injected embryos were stained using two neuronal markers that are expressed in differentiated neurons, neural specific  $\beta$ -tubulin and tanabin. *In situ* hybridization for  $\beta$ -tubulin and tanabin was carried out as described above. Over-expression of neuroD1 dramatically increased the  $\beta$ -tubulin signals in the region of the  
10 neural plate containing both motor neurons and Rohon-Beard cells at stage 14. The earliest ectopic  $\beta$ -tubulin positive cells on the injected side were observed at the end of gastrulation when the control side did not yet show any  $\beta$ -tubulin positive cells. Tanabin was also expressed in more cells in the spinal cord in the neuroD1 injected side of the embryos at stage 14. These results suggest that neuroD1 can cause  
15 premature differentiation of the neural precursors into differentiated neurons. This is a powerful indication that, when ectopically expressed or over-expressed, neuroD1 can differentiate mitotic cells into non-dividing mature neurons.

To determine if neuroD2 also was capable of inducing ectopic neuronal development in the frog, mouse neuroD2 RNA was injected into one side of a two  
20 cell *X. laevis* embryo, the uninjected side serving as a control. The neuroD2 mRNA was made from pCS2-MTmND2, an expression vector that was constructed as follows. Expression vectors were made in the pCS2+ or pCS2+MT (Turner, D.L. and H. Weintraub, *Genes & Dev.* 8:1434-1447, 1994), both contain the simian CMV promoter and the MT contains six copies of the myc epitope recognized by the 9e10  
25 monoclonal antibody (ATCC:CRL1729) cloned in-frame upstream of the insert. The 1.75 kb full length human neuroD1 cDNA (Tamimi et al., *Genomics* 34:418-421, 1996) from plasmid phcnd1-17a was cloned into the EcoRI site to make pCS2-hND1-17s (hereafter referred to as pCS2-hND1). The 1.53 kb genomic region containing the entire coding sequence of the human neuroD2 gene (described in Example 11)  
30 was cloned into the StuI-XbaI site to make pCS2-hND2-14B1 (hereafter referred to as pCS2-hND2). The mouse 1.95 kb neuroD2 cDNA was cloned into the EcoRI-XhoI sites to make pCS2-mND2-1.1.1 (hereafter referred to as pCS2-mND2). For the *myc*-tagged construct, a synthetic oligonucleotide mediated mutagenesis was used to introduce an EcoRI site adjacent to the initial ATG codon to result in the *myc*-tag  
35 and neuroD2 coding regions being in-frame to make pCS2MT-mND2.

When injected into *Xenopus laevis*, mouse neuroD2 mRNA was able to induce ectopic neuronal development as determined by immunohistochemistry with an anti-NCAM antibody. An anti-myc tag antibody, 9E10, was used to confirm that most ectodermal cells on the injected side of the frog expressed the myc-tagged mouse neuroD2 and approximately 80-90% of injected embryos stained positively with either the anti-myc or anti-NCAM antibodies. Injection of RNA encoding the human neuroD2 gene resulted in an ectopic neuronal phenotype similar to that seen with *Xenopus* neuroD1 and murine neuroD2. This demonstrates that both neuroD1 and neuroD2 can regulate the formation of neurons and that the human and mouse neuroD2 proteins are capable of functioning in the developing *Xenopus* embryo.

Developmental expression patterns suggest two distinct sub-families of neurogenic bHLH genes. *MATH1* and neuroD3 share similarity in the bHLH region and have similar temporal expression patterns, with RNA expression detected around embryonic day 10, but not persisting in the mature nervous system. *MATH-1* RNA was localized to the dorsal neural tube in 10.5-11.5 day embryos, but by birth was present only in the external granule cell layer of the cerebellum, the progenitors of the cerebellar granule cell layer (Akazawa et al., 1995). In contrast, the neuroD1, neuroD2, and *MATH2/NEX-1* genes are expressed in both differentiating and mature neurons. Northern analysis demonstrated that neuroD2 expression begins around embryonic day 11 and continues through day 16, the latest embryonic time point tested. NeuroD2 was detected in the brain of neonates as well as adult mice, with relatively equal abundance in both the cerebellum and cortex. Similar to neuroD2, the CNS expression of neuroD1 persists postnatally, as well as does its expression in the beta cells of the pancreas (Naya et al., 1995). Northern blot analysis indicated that neuroD1 expression in the adult mouse brain is most abundant in the cerebellum with lower levels in the cerebral cortex and brain stem. *NEX-1/MATH-2* gene expression is reported to occur by embryonic day 11.5 and at embryonic day 15.5 its expression is limited to the intermediate zone adjacent to the mitotically active ventricular zone, suggesting that *NEX-1/MATH2* is expressed primarily in the newly differentiating neurons at this stage (Bartholoma and Nave, 1994; Shimizu et al., 1995). In mature brain, *NEX-1/MATH-2* is expressed in neurons comprising the hippocampus, subsets of cortical neurons, and post migratory cerebellar granule cells, but the reports disagree on whether this gene is expressed in the dentate gyrus of the hippocampus. It is interesting to note that the Northern analysis of *MATH2* expression reported by Shimizu et al. (1995) shows high levels in the cerebral cortex and low levels in the

cerebellum, the opposite of the expression pattern seen for neuroD1, suggesting that these genes may also have significant differences in relative abundance in specific regions of the nervous system. Therefore, it appears that *MATH-1* and neuroD3 are expressed early in nervous system development and may have a role in either  
5 determining or expanding a population of neuronal precursors, whereas the persistent expression of neuroD1, neuroD2 and *NEX-1/MATH-2* suggest a role in initiating and maintaining expression of genes related to neuronal differentiation.

Kume et al. (*Biochem. Biophys. Res. Comm.* 219:526-530, 1996) have reported the cloning of a helix-loop-helix gene from rat brain using a strategy  
10 designed to identify genes that are expressed during tetanic stimulation of hippocampal neurons in a model of long-term-potentialiation. The gene they describe, *KW8*, is the rat homolog of the mouse and human neuroD2 gene described here. Kume et al. also describe expression in the adult brain, including the hippocampus. Subsequently, Yasunami et al. (*Biophys. Res. Comm.* 220:754-758, 1996) reported  
15 the mouse NDRF gene, which is nearly identical to *neuroD2* and demonstrates a similar expression pattern in adult brain by *in situ* hybridization.

While expression of either neuroD1 or neuroD2 in *Xenopus leavis* embryos resulted in ectopic neuronal development, it is interesting to note that neither neuroD1 nor neuroD2 was capable of converting all cell types in which it was present into  
20 neurons. As in the case of neuroD1, the ectopic neurons induced by neuroD2 were confined to a subpopulation of ectodermal cells, as indicated by the spotty NCAM positive staining pattern. The apparent restricted activity of the neuroD proteins to a subset of cells derived from the ectoderm suggests that other factors may regulate their activity, such as the *notch* pathway that mediates lateral inhibition during  
25 *Drosophila* neurogenesis.

While the induction of ectopic neurogenesis by both neuroD1 and neuroD2 in *Xenopus* embryos suggests a similar function, the developmental expression patterns and *in vitro* transfection experiments indicate that the family members may serve both  
30 overlapping and distinct functions. Previous studies have demonstrated that neuroD/beta2 and NEX-1/MATH2 can bind the core CANNTG sequence of an E-box as a heterodimer with an E-protein and activate transcription.

In the work presented here, it is shown that both neuroD1 and neuroD2 can activate a construct containing multimerized E-boxes. They also activate a construct driven by a genomic fragment from the neuroD2 gene that presumably contains  
35 regulatory regions for neuroD2, and the temporal expression pattern of neuroD1 and

neuroD2 proteins in embryogenesis and P19 differentiation suggests a model in which neuroD1 may activate neuroD2 expression during development. Most important, however, is the demonstration that neuroD1 and neuroD2 have different capacities to activate a construct driven by the core regulatory sequences of the *GAP-43* gene, demonstrating that the highly related neuroD1 and neuroD2 proteins are capable of regulating specific subsets of genes. This promoter contains several E-boxes and it remains to be determined if neuroD2 directly binds to these sites.

In the bHLH region, neuroD1 and neuroD2 differ by only 2 amino acids and it would be anticipated that they recognize the same core binding sequences. Therefore, the differential regulation of transcriptional activity may be determined independently of DNA binding. The amino acid following the histidine in the junction region of the basic region is a glycine in neuroD1, NEX-1/MATH2, and MATH1, an aspartate in neuroD2, and an asparagine in neuroD3. This residue is positioned at the same site as the lysine residue in the myogenic bHLH proteins that has been shown to be one of the critical for myogenic activity (Davis et al., *Cell* 60:733-746, 1990; Davis, R. L. and H. Weintraub, *Science* 256:1027-1030, 1992; Weintraub et al., *Genes & Dev.* 5:1377-1386, 1991). In this case, it has been postulated to be a site of potential interaction with co-activator factors that regulate transcriptional activity. If the neuroD proteins have a similar mechanism for exerting their regulatory activities, it is possible that amino acid variability in this amino acid mediates different target specificities. Alternatively, the more divergent amino- and carboxyterminal regions could confer regulation by interaction with other activators or repressors.

The different expression patterns in the mature nervous system and the subtle differences in target genes is similar to myogenic bHLH proteins. In mature muscle, *MyoD* is expressed in fast muscle fibers and *myogenin* in slow fibers (Asakura et al., *Develop. Biol.* 171:386-398, 1995; Hughes et al., *Development* 118:1137-1147, 1993) and transfection studies demonstrate that sequences adjacent to the core E-box sequence can differentially regulate the ability of MyoD and myogenin to function as transcriptional activators (Asakura et al., *Molec. & Cell. Biol.* 13:7153-7162, 1993), presumably by interaction of other regulatory factors with the non-bHLH regions of MyoD and myogenin. For the neuroD-related genes, the partially overlapping expression patterns and partially overlapping target genes suggest that they may act in a combinatorial fashion to directly regulate overlapping subsets of genes and thereby confer specific neuronal phenotypes. In this model, it is possible that a small family of neuroD-related transcription factors acts to establish the identity of a limited number



of neuronal sub-types and that local inductive events influence the generation of a higher complexity. Alternatively, it is possible that many additional members of this sub-family are yet to be identified and they may act to directly determine specific neuronal attributes.

5

### EXAMPLE 11

#### Genomic clones of human neuroD1, neuroD2 and neuroD3 and mouse neuroD3.

Genomic clones encoding human neuroD1 were obtained by probing a human fibroblast genomic library with the mouse neuroD1 cDNA. Host *E. coli* strain LE392 (New England Biolabs) were grown in LB + 10 mM MgSO<sub>4</sub>, 0.2% maltose overnight at 37°C. The cells were harvested and resuspended in 10 mM MgSO<sub>4</sub> to a final OD600 of 2. The resuspended cells were used as hosts for phage infection. The optimal volume of phage stock for use in this screening was determined by using serial dilutions of the phage stock of a human fibroblast genomic library in lambda FIX II (STRATAGENE) to infect LE392 cells (New England Biolabs). To obtain approximately 50,000 plaques per plate, a 2.5 µl aliquot of the phage stock was used to infect 600 µl of the resuspended LE392 cells. The cells were incubated with the phage for 15 minutes at 37°C, after which the cells were mixed with 6.5 ml of top agar warmed to 50°C. The top agar was plated on solid LB, and incubated overnight at 37°C. A total of 22 15-cm plates were prepared in this manner.

Duplicate plaque lifts were prepared. A first set of HYBOND membranes (Amersham) were placed onto the plates and allowed to sit for 2 minutes. The initial membranes were removed and the duplicate membranes were laid on the plates for 4 minutes. The membranes were allowed to air dry; then the phage were denatured in 0.5 M NaOH, 1.5 M NaCl for 7 minutes. The membranes were neutralized with two washes in neutralization buffer (1.5M NaCl, 0.5 M Tris, pH 7.2). After neutralization, the membranes were crosslinked by exposure to UV. A 1 kb Eco RI-Hind III fragment containing murine neuroD1 coding sequences was random primed using the Random Priming Kit (Boehringer Mannheim) according to the manufacturer's instructions. Membranes were prepared for hybridization by placing six membranes in 10 ml of FBI hybridization buffer (100 g polyethylene glycol 800, 350 ml 20% SDS, 75 ml 20X SSPE; add water to a final volume of one liter) and incubating the membranes at 65°C for 10 minutes. After 10 minutes, denatured salmon sperm DNA was added to a final concentration of 10 µg/ml and denatured probe was added to a final concentration of 0.25-0.5 x 10<sup>7</sup> cpm/ml. The membranes were hybridized at 65°C for a period of 8 hours to overnight. After incubation, the

excess probe was removed, and the membranes were washed first in 2 X SSC, 0.1% SDS for 30 minutes at 50°C. The first wash was followed by a final wash in 0.1 X SSC, 0.1% SDS for 30 minutes at 55°C (moderate stringency). Autoradiographs of the membranes were prepared. The first screen identified 55 putative positive  
5 plaques. Thirty-one of the plaques were subjected to a secondary screen using the method essentially set forth above. Ten positive clones were identified and subjected to a tertiary screen as described above. Eight positive clones were identified after the tertiary screen. Of these eight clones, three (14B1, 9F1 and 20A1) were chosen for further analysis. Clones 14B1 and 20A1 were deposited at the American Type  
10 Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852 USA, on November 1, 1995, under accession numbers 69943 and 69942, respectively.

Phage DNA was prepared from clones 14B1, 9F1, and 20A1. The 14B1 and 20A1 phage DNA were digested with Pst I to isolate the 1.2 kb and 1.6 kb fragments, respectively, that hybridized to the mouse neuroD1 probe. The 9F1 phage DNA was  
15 digested with Eco RI and SacI to obtain an approximately 2.2 kb fragment that hybridizes with the mouse neuroD1 probe. The fragments were each subcloned into plasmid BLUESCRIPT SK (STRATAGENE) that had been linearized with the appropriate restriction enzyme(s). The fragments were sequenced using SEQUENASE Version 2.0 (US Biochemical) and the following primers: the  
20 universal primer M13-21, the T7 primer, and the T3 primer.

Sequence analysis of clones 9F1 (SEQ ID NOS:8 and 9), and 14B1 (SEQ ID NOS:10 and 11) showed a high similarity between the mouse and human coding sequences at both the amino acid and nucleotide level. In addition, while clones 9F1 and 14B1 shared 100% identity in the HLH region at the amino acid level (i.e.,  
25 residues 117-156 in SEQ ID NO:9 and residues 137-176 in SEQ ID NO:11), they diverged in the amino-terminal of the bHLH. This finding strongly suggests that 14B1 is a member of the neuroD family of genes. Sequence analysis demonstrates that clone 9F1 has a high degree of homology throughout the sequence region that spans the translation start site to the end of the bHLH region. The 9F1 clone has  
30 100% identity to mouse neuroD1 in the HLH region (i.e., residues 117-156 in SEQ ID NO:9 and residues 117-156 in SEQ ID NO:2), and an overall identity of 94%. The 14B1 clone also has 100% identity to the HLH region (i.e., residues 137-176 in SEQ ID NO:11 and residues 117-156 in SEQ ID NO:2), but only 40% identity to 9F1 and 39% identity to mouse neuroD1 in the amino-terminal region. This  
35 demonstrates that 9F1 is the human homolog of mouse neuroD1, whereas the strong

conservation of the neuroD HLH identifies 14B1 as another member of the neuroD HLH subfamily. Human clone 9F1 (represented by SEQ ID NOS: 8 and 9) is referred to as human neuroD1. Human clone 14B1 is referred to as neuroD2 (SEQ ID NOS:10 and 11, and human clone 20A1 is referred to as neuroD3 (SEQ ID NOS:12 and 13).

5 A fragment of the human neuroD2 gene was used to screen both a mouse genomic library and an embryonic day 16 mouse cDNA library. An 800 bp Hind III-Eag I fragment from the neuroD2 sequences from clone 14B1 was random primed with <sup>32</sup>P, and used to screen a 16-day mouse embryo cDNA library essentially as described previously. Filters were prehybridized in FBI hybridization buffer (see above) at 50°C for 10 minutes. After prehybridization, denatured salmon sperm DNA was added to a final concentration of 10 µg/ml; denatured probe was added to a final concentration of one million cpm/ml. The filters were hybridized at 50°C overnight. After incubation, excess probe was removed, and the filters was washed first in 2 X SSC, 0.1% SDS for 30 minutes at 60°C. Genome clones were obtained and characterized. Five independent cDNAs were mapped by restriction endonucleases and demonstrated identical restriction sites and sequence. One clone, designated 1.1.1, contained 1.46 kb of murine neuroD2 cDNA as an Eco RI-Hind III insert. The nucleotide sequence and deduced amino acid sequences are shown in SEQ ID NOS:16 and 17, respectively. A comparison with the corresponding mouse genomic sequence demonstrated that the entire coding region of neuroD2 is contained in the second exon.

25 The mouse neuroD2 cDNA sequence indicated a predicted protein of 382 amino acids that differs from the major open reading frame in the human neuroD2 gene at only 9 residues, all in the aminoterminal portion of the protein. The human neuroD2 protein was found to have 98% similarity to neuroD1 and MATH2 in the bHLH region and 90% similarity in the 30 amino acids immediately carboxyterminal to the bHLH region. Similar to neuroD1 and MATH2, neuroD2 contains an aminoterminal region rich in glutamate residues that may constitute an acidic activation domain, and has other regions of similarity to neuroD1 throughout the protein.

35 Mouse neuroD3 was obtained by screening a 129SV mouse genomic library cloned in lambda-Dash II (STRATAGENE), using a labeled Pst-Pst genomic fragment containing the human neuroD coding sequence using conditions essentially as described above for selecting mouse neuroD2, with the exception that the

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prehybridization and hybridization were carried out at 55°C and the final wash was carried out at 50°C

Since all identified members of the family of genes related to neuroD1 are known to have their entire coding sequence in a single exon, the major open reading frame (ORF) encoded in the genomic DNA from human and mouse neuroD3 were  
5 determined (SEQ ID NO:12 and SEQ ID NO:21, respectively). The predicted amino acid sequences of the mouse and human neuroD3 proteins are based on the major ORF in the corresponding genomic DNAs, since cDNAs have not been cloned for these genes. The genomic sequence of mouse neuroD3 contains a major ORF of 244  
10 amino acids and the human neuroD3 gene an ORF of 237 amino acids that differs from the predicted mouse protein at 26 positions. The entire coding region of other neuroD family members is contained within a single exon, and therefore it is possible that the ORF in the neuroD3 genomic DNA represents the entire coding region, a notion supported by the conservation between mouse and human that extends to the  
15 stop codon. The major ORF predicts a smaller protein than related neuroD family members, and lacks the acidic rich aminoterminal region. The bHLH region has some elements of the loop that are similar to MATH1, but the overall level of homology in the bHLH region is closer to the neuroD-related genes. In contrast to neuroD2, the neuroD3 protein does not contain significant regions of homology to neuroD1 or  
20 MATH2/NEX-1 outside of the bHLH region and does not have an aminoterminal region rich in glutamates or acidic amino acids.

The Genbank accession numbers are: human neuroD2, U58681; mouse neuroD2, U58471; human neuroD3, U63842; mouse neuroD3, U63841.

#### EXAMPLE 12

##### 25 Chromosome mapping of human neuroD1 clones.

FISH karyotyping was performed on fixed metaphase spreads of the microcell hybrids essentially as described (Trask et al., *Am. J. Hum. Genet.* 48:1-15, 1991; and Brandriff et al., *Genomics* 10:75-82, 1991; which are incorporated by reference herein in their entirety). NeuroD1 sequences were detected using the 9F1 or 20A1 phage  
30 DNA as probes labeled using digoxigenin-dUTP (Boehringer Mannheim) according to the manufacturer's instructions. Phage DNA was biotinylated by random priming (Gibco/BRL BioNick Kit) and hybridized *in situ* to denatured metaphase chromosome spreads for 24-48 hours. Probes were detected with rhodamine-conjugated antibodies to digoxigenin, and chromosomes were counterstained with DAPI (Sigma). Signals  
35 were viewed through a fluorescence microscope and photographs were taken with

color slide film. FISH analysis indicated clone 9F1 maps to human chromosome 2q, and clone 20A1 maps to human chromosome 5.

Chromosome mapping was also carried out on a human/rodent somatic cell hybrid panel (National Institute of General Medical Sciences, Camden, NJ). This panel consists of DNA isolated from 24 human/rodent somatic cell hybrids retaining one human chromosome. For one set of experiments, the panel of DNAs were digested with Eco RI and electrophoresed on an agarose gel. The DNA was transferred to Hybond-N membranes (Amersham). A random primed (Boehringer Mannheim) 4 kb Eco RI-Sac I fragment of clone 9F1 was prepared. The filter was prehybridized in 10 ml of FBI hybridization buffer (see above) at 65°C for 10 minutes. After prehybridization, denatured salmon sperm DNA was added to a final concentration of 10 µg/ml; denatured probe was added to a final concentration of one million cpm/ml. The filter was hybridized at 65°C for a period of 8 hours to overnight. After incubation, excess probe was removed, and the filter was washed first in 2 X SSC, 0.1% SDS for 30 minutes at 65°C. The first wash was followed by a final stringent wash in 0.1 X SSC, 0.1% SDS for 30 minutes at 65°C. An autoradiograph of the filter was prepared. Autoradiographs confirmed the FISH mapping results.

In the second experiment, the panel was digested with Pst I, electrophoresed and transferred essentially as described above. A random-primed (Boehringer Mannheim) 1.6 kb Pst I fragment of clone 20A1 was prepared. The membrane was prehybridized, hybridized with the 20A1 probe and washed as described above. Autoradiographs of the Southern filter showed that 20A1 mapped to human chromosome 5 and confirmed the FISH mapping results. After autoradiography, the 20A1-probed membrane was stripped by a wash in 0.5 M NaOH, 1.5 M NaCl. The membrane was neutralized in 0.5 M Tris-HCl (pH 7.4), 1.5 M NaCl. The filter was washed in 0.1 X SSC before prehybridization. A random-primed (Boehringer Mannheim) 1.2 kb Pst I fragment of clone 14B1 was prepared. The washed membrane was prehybridized and hybridized with the 14B1 probe as described above. After washing under the previously described conditions, the membrane was autoradiographed. Autoradiographs demonstrated that clone 14B1 mapped to chromosome 17.

**EXAMPLE 13**Human neuroD1 complementary DNA.

To obtain a human neuroD1 cDNA, one million plaque forming units (pfu) were plated onto twenty LB + 10 mM MgSO<sub>4</sub> (150 mm) plates using the Stratagene human cDNA library in Lambda ZAP II in the bacterial strain XL-1 Blue (Stratagene). Plating and membrane lifts were performed using standard methods, as described in Example 11. After UV cross-linking, the membranes were pre-hybridized in an aqueous hybridization solution (1% bovine serum albumin, 1 mM EDTA, 0.5 M Na<sub>2</sub>HPO<sub>4</sub> (pH 7.4), 7% SDS) at 50°C for two hours.

The mouse neuroD1 cDNA insert was prepared by digesting the pKS+ m7a RX plasmid with Eco RI and Xho I, and isolating the fragment containing the cDNA by electroelution. A probe was made with the cDNA containing fragment by random primed synthesis with random hexanucleotides, dGTP, dATP, dTTP, alpha-<sup>32</sup>P-labeled dCTP, and Klenow in a buffered solution (25 mM Tris (pH6.9), 50 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM DTT). The probe was purified from the unincorporated nucleotides on a G-50 SEPHAROSE column. The purified probe was heat denatured at 90°C for three minutes.

After prehybridization, the denatured probe was added to the membranes in hybridization solution. The membranes were hybridized for 24 hours at 50°C. Excess probe was removed from the membranes, and the membranes were washed in 0.1 X SSC, 0.1% SDS for 20 minutes at 50°C. The wash solution was changed five times. The membranes were blotted dry and covered with plastic film before being subjected to autoradiography. Autoradiography of the filters identified 68 positive clones. The clones were plaque-purified and rescreened to obtain 40 pure, positive clones. The positive clones were screened with a random-primed Pst I fragment from clone 9F1 (human neuroD1). Twelve positive clones that hybridized with the human neuroD1 genomic probe were isolated.

The plasmid vector containing cDNA insert was excised *in vivo* from the lambda phage clone according to the STRATAGENE methodology. Briefly, eluted phage and XL-1 Blue cells (200 microliters of OD 600=1) were mixed with R408 helper phage provided by Stratagene for 15 minutes at 37°C. Five milliliters of rich bacterial growth media (2 X YT, see Sambrook et al., *ibid.*) was added, and the cultures were incubated for 3 hours at 37°C. The tubes were heated at 70°C for 20 minutes and spun for 5 minutes at 4,000 X g. After centrifugation, 200 microliters of supernatant were added to the same volume of XL-1 Blue cells (OD=1), and the

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mixture was incubated for 15 minutes at 37°C, after which the bacterial cells were plated onto LB plates containing 50 mg/ml ampicillin. Each colony was picked and grown for sequencing template preparation. The clones were sequenced and compared to the human genomic sequence. A full length cDNA encoding human neuroD1 that was identical to the 9F1 neuroD1 genomic sequence was obtained and designated HC2A. The nucleotide and deduced amino acid sequences are shown in SEQ ID NOS: 14 and 15, respectively. Clone HC2A was deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852 USA, on November 1, 1995, under accession number 69944.

Using a random-primed radiolabeled antisense probe to the mouse neuroD2 (Boehringer Mannheim), the expression pattern was determined using Northern analysis. Filters containing murine RNA from the brain and spinal cords of embryonic through adult mice were probed at high stringency and washed in 0.1 X SSC, 0.1% SDS at 65°C. Northern analysis showed neuroD2 expression in the brain and spinal cords of mice from embryonic day 12.5 through adult.

Experiments were conducted also to isolate a cDNA corresponding to mouse neuroD3 mRNA. Using procedures similar to those described above, a random-primed 1.1 kb Pst I fragment from human neuroD3 clone 20A1 was prepared and used to screen mouse embryo and newborn mouse brain libraries. For unknown reasons, no positive clones were obtained. Likewise, attempts to clone human neuroD3 cDNA have been unsuccessful. The difficulty in obtaining neuroD3 cDNA may be secondary to instability of the construct in the library, since deletions in the genomic DNA were common during amplification.

#### EXAMPLE 14

##### Construction of knock-out mice

Knock-out mice in which the murine neuroD1 coding sequence was replaced with the  $\beta$ -galactosidase gene and the neomycin resistance gene (neo) were generated i) to assess the consequences of eliminating the murine neuroD1 protein during mouse development and ii) to permit examination of the expression pattern of neuroD1 in embryonic mice. Genomic neuroD1 sequences used for these knock-out mice were obtained from the 129/Sv mice so that the homologous recombination could take place in a congenic background in 129/Sv mouse embryonic stem cells. Several murine neuroD1 genomic clones were isolated from a genomic library prepared from 129/Sv mice (Zhuang et al., *Cell* 79:875-884, 1994; which is incorporated herein by reference in its entirety) using the Bam HI-Not I neuroD1 cDNA containing fragment

of pSK+1-83 (Example 2) as a random-primed probe essentially as described in Example 11. Plasmid pPNT (Tybulewicz et al., *Cell* 65:1153-1163, 1991; which is incorporated herein by reference in its entirety) containing the neomycin resistance gene (*neo*; a positive selection marker) and the *Herpes simplex* virus thymidine kinase gene (*hsv-tk*; a negative selection marker) under the control of the PGK promoter provided the vector backbone for the targeting construct. A 1.4 kb 5' murine neuroD1 genomic fragment together with the 3 kb cytoplasmic  $\beta$ -galactosidase gene were inserted between the Eco RI and Xba I sites of the pPNT vector, and an 8 kb fragment containing the genomic 3' untranslated sequence of neuroD1 was inserted into the vector backbone between into the Xho I and Not I sites.

To prepare an Eco RI-Xba I fragment containing neuroD1 promoter sequences joined to the  $\beta$ -galactosidase gene, a 1.4 kb Eco RI(vector-derived)-Asp 718 fragment containing the 5' untranslated murine neuroD1 genomic sequence was ligated to a Hind III-Xba I fragment containing the cytoplasmic  $\beta$ -galactosidase gene such that the Asp 718 and Hind III sites were destroyed. The resulting approximately 4.4 kb Eco RI-Xba I fragment, containing the 5' neuroD1 genomic sequence (including the neuroD1 promoter) and the  $\beta$ -galactosidase gene in the same transcriptional orientation, was inserted into Eco RI-Xba I linearized pPNT to yield the plasmid pPNT/5'+ $\beta$ -gal. A neuroD1 fragment containing 3' untranslated DNA was obtained from a murine neuroD1 genomic clone that had been digested with Spe I and Not I(vector-derived) to yield an 8 kb fragment. To obtain a 5' Xho I site, the 8 kb fragment was inserted into Spe I-Not I linearized pBluescriptSK+ (Stratagene), and the resulting plasmid digested with Xho I and Not I to obtain the 8 kb neuroD1 3' genomic fragment. The Xho I-Not I fragment was inserted into Xho I-Not I linearized pPNT/5'+ $\beta$ -gal to yield the neuroD1 targeting vector. The final construct contained the 5' neuroD1 fragment, the  $\beta$ -galactosidase gene, and the 3' genomic neuroD1 fragment in the same orientation, and the *hsv-tk* and neomycin resistance genes in the opposite orientation.

The targeting construct was transfected by electroporation into mouse embryonic stem (ES) cells. A 129/Sv derived ES cell line, AK-7 described by Zhuang et al. (ibid.) was used for electroporation. These ES cells were routinely cultured on mitomycin C-treated (Sigma) SNL 76/7 cells (feeder cells) as described by McMahon and Bradley (*Cell* 62:1073-1085, 1990; which is incorporated herein by reference in its entirety) in culture medium containing high glucose DMEM supplemented with 15% fetal bovine serum (Hyclone) and 0.1  $\mu$ M  $\beta$ -mercaptoethanol. To prepare the



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targeting construct for transfection, 25 µg of the targeting construct was linearized by digestion with Not I, phenol-chloroform extracted, and ethanol precipitated. The linearized vector was then electroporated into  $1-2 \times 10^7$  AK-7 (ES) cells. The electroporated cells were seeded onto three 10-cm plates, with one plate receiving 50% of the electroporated cells and the remaining two plates each receiving 25% of the electroporated cells. After 24 hours, G418 was added to each of the plates to a final concentration of 150 µg/ml. After an additional 24 hours, gancyclovir was added to a final concentration of 0.2 µM to the 50% plate and one of the 25% plates. The third plate containing 25% of the electroporated cells was subjected to only G418 selection to assess the efficiency of gancyclovir selection. The culture medium for each plate was changed every day for the first few days, and then changed as needed after selection had occurred. After 10 days of selection, a portion of each colony was picked microscopically with a drawn micropipette, and was directly analyzed by PCR as described by Joyner, et al. (*Nature* 338:153-156, 1989; which is incorporated herein by reference in its entirety). Briefly, PCR amplification was performed as described (Kogan et al., *New England J. Med.* 317:985-990, 1987; which is incorporated herein by reference in its entirety) using 40 cycles of 93°C for 30 seconds, 57°C for 30 seconds, and 65°C for 3 minutes. To detect the wild-type allele, primers JL34 and JL36 (SEQ ID NOS:18 and 19, respectively) were used in the PCR reaction, to detect the mutant neuroD1 allele, primers JL34 and JL40 (SEQ ID NOS:18 and 20, respectively) were used in the PCR reaction. Positive colonies, identified by PCR, were subcloned into 4-well plates, expanded into 60 mm plates and frozen into 2-3 ampules.

Among the clones that were selected for both G418-resistance (positive selection for *neo* gene expression) and gancyclovir-resistance (negative selection for *hsv-tk* gene expression), 10% of the population contained correctly targeted integration of the vector into the murine neuroD1 locus (an overall 10% targeting frequency). The negative selection provided 4-8 fold enrichment for homologous recombination events.

To generate chimeric mice, each positive clone was thawed and passaged once on feeder cells. The transfected cells were trypsinized into single cells, and blastocysts obtained from C57BL/6J mice were injected with approximately 15 cells. The injected blastocysts were then implanted into pseudopregnant mice (C57BL/6J x CBA). Four male chimeras arose from the injected blastocysts (AK-71, AK-72, AK-74 and AK-75). The male chimeras AK-71 and AK-72 gave germ-line transmission at

a high rate as determined by the frequency of agouti coat color transmission to their offspring (F1) in a cross with C57BL/6J female mice. Since 50% of the agouti coat color offspring (F1) should represent heterozygous mutants, their genotypes were determined by Southern blot analysis. Briefly, genomic DNA prepared from tail biopsies was digested with Eco RI and probed with the 1.4 kb 5' genomic sequence used to make the targeting construct. This probe detects a 4 kb Eco RI fragment from the wild-type allele and a 6.3 kb Eco RI fragment from the mutant allele. Therefore, a Southern analysis would show a single 4 kb band for a wild-type mouse, 4 kb and 6.3 kb fragments for a heterozygous mouse, and a single 6.3 kb band for a homozygous mutant mouse. The resulting offspring (F1) heterozygous (+/-) mice, were mated with sibling heterozygous mice to give rise to the homozygous (-/-) mutant mice.

To study neuroD1 expression patterns in embryonic mice, chimeric mice or F1 heterozygous progeny from the chimera x C57B/6J mating were crossed with C57B/6J. Litters resulting from these crosses were harvested from pregnant females and stained for  $\beta$ -galactosidase activity. The embryos were dissected away from all the extra-embryonic tissue and the yolk sac was reserved for DNA analysis. The embryos were fixed for one hour in a fixing solution (0.1 M phosphate buffer containing 0.2% glutaraldehyde, 2% formaldehyde, 5 mM EGTA (pH 7.3), 2 mM  $\text{MgCl}_2$ ). The fixing solution was removed by three thirty-minute rinses with rinse solution (0.1 M phosphate buffer (pH 7.3) containing 2 mM  $\text{MgCl}_2$ , 0.1% sodium deoxycholate, 0.2% NP-40). The fixed embryos were stained overnight in the dark in rinse solution containing 1 mg/ml X-gal, 5 mM sodium ferricyanide, 5 mM sodium ferrocyanide. After staining, the embryos were rinsed with PBS and stored in the fixing solution before preparation for examination. Examination of stained tissue from fetal and postnatal mice heterozygous for the mutation confirmed the neuroD1 expression pattern in neuronal cells demonstrated previously by *in situ* hybridization (Example 4), and also demonstrated neuroD1 expression in the pancreas and gastrointestinal tract.

Blood glucose levels were detected using PRECISION QID blood glucose test strips and a PRECISION QID blood glucose sensor (Medisens Inc., Waltham, MA) according to the manufacturer's instruction. A tissue sample was taken for DNA analysis and the pups were fixed for further histological examination. Blood glucose levels in mice homozygous for the mutation (neuroD1) had blood glucose levels between 2 and 3 times higher than the blood glucose level of wild-type mice.

Heterozygous mutants exhibited similar blood glucose levels as wild-type mice. Mice that were homozygous for the mutation (lacking neuroD1) had diabetes as demonstrated by high blood glucose levels and died by day four; some homozygous mice died at birth.

#### EXAMPLE 15

##### NeuroD1 expression and activity in PC12 and P19 embryonic carcinoma cells

Murine PC12 pheochromacytoma cells differentiate into neurons in tissue culture in the presence of appropriate inducers, i.e., nerve growth factor. Neither induced nor non-induced murine PC12 cells expressed neuroD1 transcripts, nor did control 3T3 fibroblasts produce detectable levels of neuroD1 transcription products.

P19 cells are a well characterized mouse embryonic carcinoma cell line with the ability to differentiate into numerous cell types, including skeletal and cardiac muscle, or neurons and glia following treatment with dimethylsulfoxide (DMSO) or retinoic acid (RA) (Jones-Villeneuve et al., *Molec. & Cell. Biol.* 3:2271-2279, 1983), respectively. To determine whether P19 cells expressed endogenous neuroD genes during neuronal differentiation, RNA expression was analyzed for neuroD1, neuroD2, and neuroD3 in both uninduced and induced P19 cells. To induce the formation of neurons, P19 cells were cultured as aggregates in Petri dishes in the presence of retinoic acid for four days. The aggregates were then plated into tissue culture dishes in the absence of retinoic acid and neuronal differentiation occurred during a five day period, as evidenced by the formation of neurofilament positive process bearing cells.

NeuroD1 mRNA was most abundant after the cells were aggregated and treated with RA for 4 days, and continued to be expressed at decreased levels during the period of neuronal differentiation. NeuroD2 was not detected during the period of RA induction, but became abundant during the period of neuronal differentiation. Both neuroD1 and neuroD2 signals were modestly enhanced when the differentiated P19 cultures were grown in the presence of Ara-C which eliminates some of the non-neuronal dividing cells, suggesting that the neuroD1 and neuroD2 genes are preferentially expressed in the post-mitotic cell population. NeuroD3 was first detected after two days of induction, and was most abundant after 4 days of induction), however, unlike neuroD1, neuroD3 mRNA was not detected at the later, more differentiated, time points. Therefore, the temporal expression pattern of neuroD1, neuroD2, and neuroD3 in differentiating P19 cells was similar to that seen during embryonic development: a peak of neuroD3 expression at the time of neuronal commitment and early neurogenesis, early and persistent expression of neuroD1, and

slightly later and persistent expression of neuroD2. Hence, P19 cells are potentially useful in screening assays for identifying inducers of neuroD1 expression that may stimulate nerve regeneration and differentiation of neural tumor cells.

NeuroD1 and neuroD2 are both expressed in neurons and both can induce neurogenesis when expressed in frog embryos. To determine if they have the ability to activate similar target genes, expression vectors were constructed driving the human neuroD1 or neuroD2 coding regions from a simian cytomegalovirus promoter; these vectors are pCS2-hND1 and pCS2-hND2, whose construction is described in Example 10. The activity of neuroD1 and neuroD2 was assayed on reporter constructs co-transfected into P19 cells. Other members of the neuroD family have been shown to bind consensus E-box sequences *in vitro*. Gel shift assays have demonstrated that MATH-1 and NEX-1/MATH-2 bind the consensus E-box site CAGGTG as a heterodimer with the E47 protein, and activate the transcription of reporter constructs (Akazawa et al., *J. Biol. Chem.*, 270:8730-8738, 1995; Bartholoma and Nave, 1994; Shimizu et al., *Eur. J. Biochem.*, 229:239-248, 1995). *In vitro* gel shift assays demonstrated that neuroD1 and neuroD2 proteins can bind to an oligo containing the core E-box CACCTG as a heterodimer with an E-protein. Therefore, neuroD1 and neuroD2 proteins were tested for the ability to activate transcription of the reporter construct P4RTK-luc, which is composed of a multimerized E-box with the same core sequence and the minimal promoter from the thymidine kinase gene driving the reporter gene *luciferase*.

Prior to transfection, P19 cells were cultured in minimal essential medium alpha supplemented with 10% fetal bovine serum. Transfections were performed as previously described (Tamura, M. and M. Noda, *J. Cell Biol.* 126: 7731994), using BBS calcium chloride precipitation. Forty-eight hours after transfection, the cells were harvested and assayed for luciferase and lacZ. Construction of the expression vectors pCS2-hND1 and pCS2-hND2 were as described in Example 10. The pGAP43-luc construct, a neuronal specific promoter construct that is upregulated *in vivo* in post-mitotic, terminally differentiating neurons (Nedivi et al., *J. Neurosci.* 12:691-704, 1992), contained the 760 base pair promoter region driving *luciferase* in a pGL2 vector modified to contain a poly-A site upstream of the multiple cloning site. The p4RTK-luc construct was made by placing the 4RTK region from HindIII to XhoI of the p4RTK-CAT vector (Weintraub et al., *Proc. Natl. Acad. Sci.* 87:5623-5627, 1990) into the promoterless luciferase vector. Luciferase assays were

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performed according to Current Protocols in Molecular Biology (Brasier, A. R., John Wiley & Sons, New York, 1989, which is hereby incorporated by reference).

When P19 cells were transfected as described above, it was observed that cotransfection with either pCS2-ND1 or pCS2-ND2 modestly increased the level of activity from p4RTK-luc in P19 cells between two and four-fold.

Additional reporter constructs were tested in P19 cells to determine whether the neuroD and neuroD2 proteins had different transcriptional activation potentials. Tests were conducted to determine the ability of pCS2-ND1 and pCS2-ND2 to transactivate the luciferase reporter construct, pGAP43-luciferase. In contrast to the simple E-box driven reporter, pCS2-ND1 did not show significant transactivation of the pGAP43-luciferase, while pCS2-ND2 induced expression from this construct by approximately 4-fold over the basal activity.

The myogenic bHLH proteins show auto- and cross-regulation, and expression of NEX-1/MATH-2 has been shown to activate a reporter driven by the *NEX-1/MATH-2* promoter (Bartholoma and Nave, 1994). To determine if neuroD1 or neuroD2 could activate a construct containing the neuroD2 promoter, an expression vector was constructed that contained a one kilobase fragment upstream of the mouse neuroD2 gene, terminating in the first exon, driving the luciferase reporter gene. P19 cells were co-transfected with this pND2-luc reporter construct and either of the neuroD expression vectors. Both pCS2-ND1 and pCS2-ND2 transactivated this reporter construct, suggesting that neuroD2 may be auto-regulated and cross-regulated by other members of the neuroD family, in a manner analogous to the regulation of the myogenic bHLH genes.

Together these transfection experiments demonstrate that neuroD1 and neuroD2 proteins can both activate some target genes, such as a multimerized E-box reporter and the neuroD2 promoter; whereas the reporter construct driven by the *GAP43* promoter seems to be preferentially activated by neuroD2.

#### EXAMPLE 16

##### In situ localization of neuroD1 and neuroD2 RNA in adult mouse brain

To address whether neuroD1 and neuroD2 were expressed in neurons in the adult mouse brain and whether they were expressed in the same cells, *in situ* hybridizations were performed using <sup>35</sup>S-UTP labeled RNA probes. Sections of adult mouse brain were hybridized to anti-sense probes derived from the mouse neuroD1 and neuroD2 cDNA fragments using T3 and T7 generated transcripts for sense and anti-sense probes, and incorporating <sup>35</sup>S-UTP label. Frozen 4-5 micron sagittal

sections of adult mouse brain were cut, placed on Fisher SUPERFROST slides (Fisher Scientific, Tustin, CH), and frozen at -80°C. Hybridization to <sup>35</sup>S-UTP labeled probes and autoradiography was performed according to Masters et al. (*J. Neurosci.* 14:5844-5857, 1994, which is hereby incorporated by reference). After washing to  
5 remove unhybridized probe, sections were coated with liquid photographic emulsion. After development of the emulsion, dark field optics illuminated the silver grains as white spots at magnification X160.

In the cerebellum, *neuroD1* was easily detected in the granule layer, whereas the *neuroD2* signal was less intense in this region and was largely restricted to the  
10 region of the Purkinje cells. In contrast, the *neuroD1* and *neuroD2* signals in the pyramidal cells and dentate gyrus of the hippocampus were easily detected. The *neuroD2* probe hybridized preferentially to the region of the Purkinje cell layer. These results demonstrate that *neuroD1* and *neuroD2* are expressed in neuronal populations in the mature nervous system, and that their relative level of expression  
15 varies among neuronal populations.

#### EXAMPLE 17

##### Neurogenic bHLH genes in human tumor specimens and in neuroectodermal tumor cell lines

Northern blot analysis was used to evaluate expression of *neuroD* family  
20 members and *hash1* in surgical specimens from 13 medulloblastomas, five supratentorial PNETs, six pediatric gliomas, and cell lines derived from 14 neuroectodermal tumors and three gliomas. For these analyses, RNA extraction, gel electrophoresis, transfer to membranes, and hybridization were carried out according to standard protocols (e.g., Sambrook et al.). Hybridization was conducted under  
25 stringent conditions in FBI buffer (described in Example 11). Hybridizations were incubated for a minimum of 5 hours at 65-70°C, and filters were washed in 0.1 x SSC, 0.1% SDS at 55-63°C, depending on the probe. Probes were derived from regions outside the conserved HLH domains in order to avoid cross-hybridization among genes. None of the probes used in these experiments cross-reacted with RNA from  
30 other family members under these hybridization conditions. *In situ* hybridization analysis was used to confirm data obtained by Northern blot analysis, and to determine whether expression of these genes was ubiquitous in tumor cells.

Human tumor tissue samples were obtained at craniotomy. Samples were either snap frozen in liquid nitrogen and stored at -80°C, or were placed in tissue  
35 culture medium, mechanically disassociated, filtered, and the cell pellet collected by

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centrifugation. The cell lines analyzed for expression of neurogenic bHLH genes are listed in Table 1. Cell lines were obtained from the American Type Culture Collection (ATCC) except for: NLF, NGP, and NMB, which were obtained from G. Brodeur, University of Pennsylvania, and UW228, T9AG and SNB19, which were obtained  
5 from J. Silver and M. Berger, University of Washington. Y79, WERI, H2O9 and H82 cells were grown in RPMI 1640 (GIBCO) with 10% FBS (HYCLONE). The remainder were grown in Eagles MEM with Earle's BSS, 1x non-essential amino acids (GIBCO), 1 mM pyruvate (GIBCO) and 10% FBS or FCS.

Total RNA was extracted from confluent cell lines and tissue specimens or cell  
10 pellets using TRIZOL reagent (GIBCO). Northern analysis was performed according to standard protocols with minor modifications (Bennahmias, S., *American Biotech Lab* 7(8):10-12, 1989; Chin, M.S. et al., *J. Comparative Neurol.* 349:389-400, 1994). Because amounts of the surgical specimens were limited, neuroD2 or neuroD3 expression was determined for some specimens on stripped membranes that  
15 had previously been used for hybridization with probes corresponding to neuroD1 or hash1 expression, respectively. To ensure that previously applied probes had been adequately removed from the stripped filters, the filters were reprobbed only after negative results were obtained in a four-day exposure to x-ray film. Human neuroD1 messenger RNA was detected using either a full length 1.6 kB cDNA probe (SEQ ID  
20 NO:14), or an 800 base pair probe from a region to the 3' side of the bHLH domain, and which was isolated by Kpn digestion (Lee, J.E. et al., *Science* 268:836-844, 1995). The probe used to detect expression of human neuroD2 spanned 500 base pairs in the region 3' of the bHLH coding region and located between Pst I and Sac I excision sites (McCormick et al., *Mol. Cell Biol.* 16:5792-5800, 1996). The human  
25 neuroD3 probe spanned 800 base pairs from the Sma I to Pst I restriction sites of the clone SK20A1 (McCormick et al., 1996). Hash1 probes spanned the bHLH domain and were obtained using PCR primers 5'GTCACAAGTCAGCGCCCAAG 3' (SEQ ID NO:23) and 5'CGACGAGTAGGATGAGACCG 3' (SEQ ID NO:24). Hash1 findings were confirmed using a probe derived from a 0.9 kb fragment isolated  
30 from a human fetal cDNA library (STRATAGENE) that was homologous to the published sequence in the 3' untranslated region of this cDNA (Ball et al., *Proc. Natl. Acad. Sci. USA* 90:5648-5652, 1993). None of the probes cross-reacted with RNA from other family members under the hybridization conditions used.

The results of Northern blot analyses are summarized in Table 1. In Table 1,  
35 "nd" indicates that the sample was not tested for expression in that sample. Because

of the small size of the samples, it was not possible to assay all of the samples for all four genes in this study.

Table 1. NEUROD Genes and HASH1 in Medulloblastoma

Patient	Age	Sex	Distant		NEUROD1	NEUROD2	NEUROD3	HASH1
			Metastasis					
1	5	M	Yes		+	-	+	-
2	7	M	No		+	+	-	-
3	9	M	Yes		+	+	+	-
4	10	M	No		+	+	-	-
5 <sup>a</sup>	10	M	No		+	-	+	-
6	1	F	No		+	+	-	-
7	7	M	No		+	-	-	-
8	13	M	Yes		+	nd	+	-
9	7	F	Yes		nd	nd	+	-
10	9	M	No		+	+	nd	-
11	8	M	No		+	-	nd	-
12	6	M	No		+	-	nd	-
13	3	M	No		+	nd	nd	-

5 <sup>a</sup>Atypical histology, tumor progressed early in treatment.

The results indicated that neuroD1 was expressed in all 12 medulloblastoma surgical specimens analyzed with this probe. Specimens 2, 3, 4, 6, and 10 also expressed neuroD2.

10 Nine of the surgical samples were analyzed for neuroD3 expression (samples 1-9 in Table 1). Of these, five expressed neuroD3. Four of the patients whose tumors expressed neuroD3 presented with disseminated disease, and the fifth (patient #5) developed clinical and radiographic progression in the three-week interval between tumor resection and initiation of radiation therapy. The association observed between neuroD3 expression and medulloblastoma dissemination was significant using the Fisher exact test. These observations suggested that the expression of neuroD3 in a medulloblastoma indicates an aggressive form of the tumor.

15 For conducting the *in situ* hybridizations, paraformaldehyde-fixed frozen tumor specimens were sectioned at 12 micron intervals then pretreated with 4% Proteinase K, acidic anhydride and 0.1% TRITON X-100 (neuroD1) or 4%  
20 Proteinase K and acidic anhydride (neuroD2 and neuroD3). *In situ* analysis was



performed according to published procedure except that the concentration of NBT was reduced ten-fold for neuroD2 and neuroD3 assays (Schaeran-Wiemers and Gerfin-Moser, *Histochem.* 100:431-440, 1993, which is hereby incorporated by reference).

5        *In situ* hybridization using probes corresponding to neuroD1, neuroD2 and neuroD3 were conducted with medulloblastoma samples. When antisense probes were used robust staining was observed in the characteristically scant cytoplasm, whereas background hybridization obtained with sense probes was minimal. In tumors that expressed neuroD1, nearly all the cells were positive, a result consistent  
10 with the homogeneous nature of medulloblastomas. Expression of neuroD1 and neuroD3 in esthesioneuroblastoma samples, a rare type of neuroblastoma, when assayed by *in situ* analysis demonstrated patchy expression, suggesting that expression may be more heterogeneous in some types of neuroblastsoma tumors than in others.

Medulloblastoma samples probed with a human clone of hash1 (Ball et al.,  
15 1993) were uniformly negative when analyzed by Northern blot analysis. In contrast, three of five supratentorial PNETs expressed hash1. These results suggest that while medulloblastoma and cerebral PNETs are similar histologically, they probably derive from distinct progenitor cell populations, and they can be differentiated by analyzing tumor samples for hash1 expression.

20        Six pediatric gliomas, including cerebellar pilocytic astrocytoma, cerebral pilocytic astrocytoma, ependymoma and malignant astrocytoma specimens as well as three human glioma cell lines failed to express any of the neurogenic bHLH messenger RNAs when analyzed by Northern blot with probes corresponding to neuroD1, neuroD2, and neuroD3.

25        Cell lines derived from various neuroectodermal tumors were analyzed to further elucidate the patterns of neurogenic bHLH expression. Northern blot analysis was performed on 13 cell lines derived from tumors of presumed neuroectodermal origin. Consistent with the above-described analysis of patient samples, the metastatic medulloblastoma cell lines D283 and D341 expressed neuroD1 and neuroD3 (see  
30 Table 2). These observations suggest that co-detection of neuroD1 and neuroD3 expression products is a means of distinguishing medulloblastoma from other types of PNETs.

Table 2. NEUROD Genes and HASH1 in Cell Lines

	NeuroD1	NeuroD2	NeuroD3	Hash1
<u>Medulloblastoma:</u>				
D283	+	-	+	-
D341	+	-	+	-
UW228	-	-	-	-
DAOY	-	-	-	-
<u>Neuroblastoma:</u>				
SKN-SH	+	-	-	+
NLF	-	-	-	-
NGP	+	-	-	+
NMB	-	-	-	+
IMR 32	+	-	-	+
SKN-MC	-	-	-	-
<u>Retinoblastoma:</u>				
Y79	+	-	+	-
WERI	+	-	-	-
<u>Small Cell Lung Cancer:</u>				
H82	+	-	+	-
H209	-	-	+	+
<u>Glioma:</u>				
SNB 19	-	-	-	-
T98G	-	nd	nd	-
SF767	-	-	-	-

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While the medulloblastoma cell lines did not express hash1, a gene critical for sympathoadrenal lineage development, it is notable that the neuroblastoma cell lines SKN-SH, NGP, NMB and IMR 32 did express this gene. (Guillemot et al., *Cell* 75:463-476, 1993). A number of neuroblastoma cell lines, i.e., SKN-SH, NGP, and  
5 IMR32, coexpressed hash1 and neuroD1. NeuroD2 and *neuroD3* expression was not detected in any of the neuroblastoma cell lines that were analyzed. These observations suggest that neuroblastoma can be distinguished from other types of brain tumors by the co-detection of hash1 and neuroD1 expression.

No neuroD mRNAs were detected by Northern blot analysis in DAOY and  
10 UW228 medulloblastoma cells or in NLF and SKN-MC neuroblastoma cells. However, these cell lines may express bHLH proteins other than those tested (e.g., atonal homologs), or expression in these cells may be below the limit of detection by Northern blot analysis.

Retinoblastoma cell lines also were analyzed by Northern blot analysis. These  
15 were observed to express neuroD genes but not hash1. Y79, a cell line derived from familial retinoblastoma, expressed neuroD1 and neuroD3. WERI, a spontaneous retinoblastoma cell, expressed neuroD1 but not any of the others for which expression was evaluated. NeuroD1 is expressed in developing and mature retina, but expression in normal retina of neuroD2 and neuroD3 has not been reported. *Achaete-scute*  
20 homologs are expressed in late but not early retinal progenitors (Jasoni et al., *Development* 120:769-783, 1994; Jasoni and Reh, *J. Comparative Neurol.* 369:319-327, 1996). These data suggest that the retinoblastomas from which Y79 and WERI cell lines were derived from early retinal progenitors.

The lineage from which small cell lung cancers arise remains controversial.  
25 This type of cancer cell expresses proteins that normally are found in the central nervous system. One aspect of small cell lung cancer is that the patient's body frequently develops an autoimmune reaction against the cancer cells, and the resulting self-antibodies may subsequently attack the brain itself. Two small cell lung cancer cell lines, H82 and H209, were analyzed by Northern blot as described above for  
30 bHLH gene expression. It was observed that H82 cells expressed neuroD1 and neuroD3, and H209 cells expressed hash1 and neuroD3 (see Table 2). Possible explanations for these observations are that neurogenic bHLH factors are expressed because these tumors arise from neuroendocrine cells, or that neuroD/*achaete-scute* genes are deregulated in these tumors, thus conferring them with a neuronal  
35 phenotype although they were derived from a nonneuronal lineage.

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NeuroD2 was not expressed in any neuroectodermal tumor cell line tested. As several of the tumors analyzed had expressed neuroD2 (see Table 1), this result was unexpected. It is possible that none of the cell lines tested were derived from neuroD2-expressing tumors. Alternatively, neuroD2 expression might confer a growth disadvantage to cells so that they are selected against in culture.

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modification may be made without deviating from the spirit and scope of the invention.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A method of classifying a human neuroectodermal tumor by analyzing a sample of the tumor comprising:

measuring the expression of at least one basic helix loop helix gene in the sample; and

determining that the tumor belongs to a subclass of neuroectodermal tumor if the basic helix loop helix gene expressed in the sample corresponds to a predetermined profile of basic helix loop helix expression associated with the subclass of neuroectodermal tumor.

2. The method of Claim 1, wherein the basic helix loop helix gene is selected from among the group consisting of neuroD1, neuroD2, and neuroD3.

3. The method of Claim 1, wherein the expression of neuroD1, neuroD2, and neuroD3 genes is measured in the sample.

4. A method of classifying a human neuroectodermal tumor as a medulloblastoma by analyzing a sample of the tumor comprising:

measuring neuroD1 and neuroD3 expression in the sample; and

determining that the neuroectodermal tumor is a medulloblastoma if both neuroD1 and neuroD3 expression are detected in the sample.

5. A method of prognosticating a human medulloblastoma by analyzing a sample of the tumor comprising:

measuring neuroD3 expression in the sample; and

determining that the neuroectodermal tumor is an aggressive medulloblastoma if neuroD3 expression is detected in the sample.

6. The method of Claim 5, wherein the step of measuring neuroD3 expression comprises:

hybridizing RNA from the sample with a nucleic acid probe corresponding to a non-conserved region of neuroD3 and that is capable of hybridizing under stringent conditions with the nucleotide sequence shown in SEQ ID NO:12; and

-60-

determining that neuroD3 is expressed in the sample if duplexes between the RNA and the nucleic acid probe are detected in the product of the hybridization reaction.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANTS: Tapscott, Stephen J.  
Olson, James M.
- (ii) TITLE OF INVENTION: Expression of Neurogenic bHLH Genes in Primitive Neuroectodermal Tumors
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- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
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  - (B) FILING DATE: 05-August-1998
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  - (A) APPLICATION NUMBER: WO PCT/US95/05741
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- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: PCT/US96/17532
  - (B) FILING DATE: 30-October-1996
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER: US 08/910,973
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## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 2089 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Mus musculus
- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) LOCATION: 229..1302
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```

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AGGCATCCAT TTTGCAGTGG ACTCCTGTGT ATTTCTATTT GTGTGCATTT CTGTAGGATT      120
AGGGAGAGGG AGCTGAAGGC TTATCCAGCT TTAAATATA GCGGGTGGAT TTCCCCCCT      180
TTCTTCTTCT GCTTGCTCT CTCCCTGTTC AATACAGGAA GTGGAAAC ATG ACC AAA      237
                                         Met Thr Lys

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TCA TAC AGC GAG AGC GGG CTG ATG GGC GAG CCT CAG CCC CAA GGT CCC Ser Tyr Ser Glu Ser Gly Leu Met Gly Glu Pro Gln Pro Gln Gly Pro 5 10 15	285
CCA AGC TGG ACA GAT GAG TGT CTC AGT TCT CAG GAC GAG GAA CAC GAG Pro Ser Trp Thr Asp Glu Cys Leu Ser Ser Gln Asp Glu Glu His Glu 20 25 30 35	333
GCA GAC AAG AAA GAG GAC GAG CTT GAA GCC ATG AAT GCA GAG GAG GAC Ala Asp Lys Lys Glu Asp Glu Leu Glu Ala Met Asn Ala Glu Glu Asp 40 45 50	381
TCT CTG AGA AAC GGG GGA GAG GAG GAG GAG GAA GAT GAG GAT CTA GAG Ser Leu Arg Asn Gly Gly Glu Glu Glu Glu Glu Asp Glu Asp Leu Glu 55 60 65	429
GAA GAG GAG GAA GAA GAA GAG GAG GAG GAG GAT CAA AAG CCC AAG AGA Glu Glu Glu Glu Glu Glu Glu Glu Glu Asp Gln Lys Pro Lys Arg 70 75 80	477
CGG GGT CCC AAA AAG AAA AAG ATG ACC AAG GCG CGC CTA GAA CGT TTT Arg Gly Pro Lys Lys Lys Lys Met Thr Lys Ala Arg Leu Glu Arg Phe 85 90 95	525
AAA TTA AGG CGC ATG AAG GCC AAC GCC CGC GAG CGG AAC CGC ATG CAC Lys Leu Arg Arg Met Lys Ala Asn Ala Arg Glu Arg Asn Arg Met His 100 105 110 115	573
GGG CTG AAC GCG GCG CTG GAC AAC CTG CGC AAG GTG GTA CCT TGC TAC Gly Leu Asn Ala Ala Leu Asp Asn Leu Arg Lys Val Val Pro Cys Tyr 120 125 130	621
TCC AAG ACC CAG AAA CTG TCT AAA ATA GAG ACA CTG CGC TTG GCC AAG Ser Lys Thr Gln Lys Leu Ser Lys Ile Glu Thr Leu Arg Leu Ala Lys 135 140 145	669
AAC TAC ATC TGG GCT CTG TCA GAG ATC CTG CGC TCA GGC AAA AGC CCT Asn Tyr Ile Trp Ala Leu Ser Glu Ile Leu Arg Ser Gly Lys Ser Pro 150 155 160	717
GAT CTG GTC TCC TTC GTA CAG ACG CTC TGC AAA GGT TTG TCC CAG CCC Asp Leu Val Ser Phe Val Gln Thr Leu Cys Lys Gly Leu Ser Gln Pro 165 170 175	765
ACT ACC AAT TTG GTC GCC GGC TGC CTG CAG CTC AAC CCT CGG ACT TTC Thr Thr Asn Leu Val Ala Gly Cys Leu Gln Leu Asn Pro Arg Thr Phe 180 185 190 195	813
TTG CCT GAG CAG AAC CCG GAC ATG CCC CCG CAT CTG CCA ACC GCC AGC Leu Pro Glu Gln Asn Pro Asp Met Pro Pro His Leu Pro Thr Ala Ser 200 205 210	861
GCT TCC TTC CCG GTG CAT CCC TAC TCC TAC CAG TCC CCT GGA CTG CCC Ala Ser Phe Pro Val His Pro Tyr Ser Tyr Gln Ser Pro Gly Leu Pro 215 220 225	909
AGC CCG CCC TAC GGC ACC ATG GAC AGC TCC CAC GTC TTC CAC GTC AAG Ser Pro Pro Tyr Gly Thr Met Asp Ser Ser His Val Phe His Val Lys 230 235 240	957
CCG CCG CCA CAC GCC TAC AGC GCA GCT CTG GAG CCC TTC TTT GAA AGC Pro Pro Pro His Ala Tyr Ser Ala Ala Leu Glu Pro Phe Phe Glu Ser 245 250 255	1005
CCC CTA ACT GAC TGC ACC AGC CCT TCC TTT GAC GGA CCC CTC AGC CCG	1053



Pro 260	Leu	Thr	Asp	Cys	Thr 265	Ser	Pro	Ser	Phe	Asp 270	Gly	Pro	Leu	Ser	Pro 275	
CCG	CTC	AGC	ATC	AAT	GGC	AAC	TTC	TCT	TTC	AAA	CAC	GAA	CCA	TCC	GCC	1101
Pro	Leu	Ser	Ile	Asn 280	Gly	Asn	Phe	Ser	Phe 285	Lys	His	Glu	Pro	Ser	Ala 290	
GAG	TTT	GAA	AAA	AAT	TAT	GCC	TTT	ACC	ATG	CAC	TAC	CCT	GCA	GCG	ACG	1149
Glu	Phe	Glu	Lys	Asn 295	Tyr	Ala	Phe	Thr	Met	His	Tyr	Pro	Ala	Ala	Thr 300	
CTG	GCA	GGG	CCC	CAA	AGC	CAC	GGA	TCA	ATC	TTC	TCT	TCC	GGT	GCC	GCT	1197
Leu	Ala	Gly 310	Pro	Gln	Ser	His 315	Gly	Ser	Ile	Phe	Ser	Ser 320	Gly	Ala	Ala	
GCC	CCT	CGC	TGC	GAG	ATC	CCC	ATA	GAC	AAC	ATT	ATG	TCT	TTC	GAT	AGC	1245
Ala	Pro	Arg	Cys	Glu	Ile	Pro 325	Ile	Asp	Asn	Ile	Met	Ser	Phe	Asp	Ser 330	
CAT	TCG	CAT	CAT	GAG	CGA	GTC	ATG	AGT	GCC	CAG	CTT	AAT	GCC	ATC	TTT	1293
His	Ser	His	His	Glu	Arg	Val 340	Met	Ser	Ala	Gln 345	Leu	Asn	Ala	Ile	Phe 350	
CAC	GAT	TAGAGGGCAC	GTCAAGTTTCA	CTATTCCTGG	GAAACGAATC	CACTGTGCGT										1349
His	Asp															
ACAGT	GA	CTG	TCCTG	TTTAC	AGAAGGCAGC	CCTTTTGCTA	AGATTGCTGC	AAAGTGCAAA								1409
TACTCAAAGC	TTCAAGTGAT	ATATGTATTT	ATTGTCGTTA	CTGCCTTTGG	AAGAAACAGG											1469
GGATCAAAGT	TCCTGTTTAC	CTTATGTATT	GTTTTCTATA	GCTCTTCTAT	TTTAAAAATA											1529
ATAATACAGT	AAAGTAAAAA	AGAAAATGTG	TACCACGAAT	TTCGTGTAGC	TGTATTTCAGA											1589
TCGTATTAAT	TATCTGATCG	GGATAAAAAA	AATCACAAGC	AATAATTAGG	ATCTATGCAA											1649
TTTTTTAACT	AGTAATGGGC	CAATTAAAAT	ATATATAAAT	ATATATTTTT	CAACCAGCAT											1709
TTTACTACCT	GTGACCTTTC	CCATGCTGAA	TTATTTTGTT	GTGATTTTGT	ACAGAATTTT											1769
TAATGACTTT	TTATAACGTG	GATTTCTTAT	TTTAAACCA	TGCAGCTTCA	TCAATTTTTTA											1829
TACATATCAG	AAAAGTAGAA	TTATATCTAA	TTTATACAAA	ATAATTTAAC	TAATTTAAAC											1889
CAGCAGAAAA	GTGCTTAGAA	AGTTATTGCG	TTGCCTTAGC	ACTTCTTTCT	TCTCTAATTG											1949
TAAAAAAGAA	AAAAAAAAAA	AAAAAACTCG	AGGGGGGGCC	CGGTACCCAG	CTTTTGTTCC											2009
CTTTAGTGAG	GGTTAATTGC	GCGCTTGCG	TAATCATGGT	CATAGCTGTT	TCCTGTGTGA											2069
ATTGTTATCC	GCTCACAATT															2089

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 357 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Thr	Lys	Ser	Tyr	Ser	Glu	Ser	Gly	Leu	Met	Gly	Glu	Pro	Gln	Pro
1					5				10					15	
Gln	Gly	Pro	Pro	Ser	Trp	Thr	Asp	Glu	Cys	Leu	Ser	Ser	Gln	Asp	Glu
		20						25					30		

Glu His Glu Ala Asp Lys Lys Glu Asp Glu Leu Glu Ala Met Asn Ala  
 35 40 45  
 Glu Glu Asp Ser Leu Arg Asn Gly Gly Glu Glu Glu Glu Glu Asp Glu  
 50 55 60  
 Asp Leu Glu Glu Glu Glu Glu Glu Glu Glu Glu Asp Gln Lys  
 65 70 75 80  
 Pro Lys Arg Arg Gly Pro Lys Lys Lys Lys Met Thr Lys Ala Arg Leu  
 85 90 95  
 Glu Arg Phe Lys Leu Arg Arg Met Lys Ala Asn Ala Arg Glu Arg Asn  
 100 105 110  
 Arg Met His Gly Leu Asn Ala Ala Leu Asp Asn Leu Arg Lys Val Val  
 115 120 125  
 Pro Cys Tyr Ser Lys Thr Gln Lys Leu Ser Lys Ile Glu Thr Leu Arg  
 130 135 140  
 Leu Ala Lys Asn Tyr Ile Trp Ala Leu Ser Glu Ile Leu Arg Ser Gly  
 145 150 155 160  
 Lys Ser Pro Asp Leu Val Ser Phe Val Gln Thr Leu Cys Lys Gly Leu  
 165 170 175  
 Ser Gln Pro Thr Thr Asn Leu Val Ala Gly Cys Leu Gln Leu Asn Pro  
 180 185 190  
 Arg Thr Phe Leu Pro Glu Gln Asn Pro Asp Met Pro Pro His Leu Pro  
 195 200 205  
 Thr Ala Ser Ala Ser Phe Pro Val His Pro Tyr Ser Tyr Gln Ser Pro  
 210 215 220  
 Gly Leu Pro Ser Pro Pro Tyr Gly Thr Met Asp Ser Ser His Val Phe  
 225 230 235 240  
 His Val Lys Pro Pro Pro His Ala Tyr Ser Ala Ala Leu Glu Pro Phe  
 245 250 255  
 Phe Glu Ser Pro Leu Thr Asp Cys Thr Ser Pro Ser Phe Asp Gly Pro  
 260 265 270  
 Leu Ser Pro Pro Leu Ser Ile Asn Gly Asn Phe Ser Phe Lys His Glu  
 275 280 285  
 Pro Ser Ala Glu Phe Glu Lys Asn Tyr Ala Phe Thr Met His Tyr Pro  
 290 295 300  
 Ala Ala Thr Leu Ala Gly Pro Gln Ser His Gly Ser Ile Phe Ser Ser  
 305 310 315 320  
 Gly Ala Ala Ala Pro Arg Cys Glu Ile Pro Ile Asp Asn Ile Met Ser  
 325 330 335  
 Phe Asp Ser His Ser His His Glu Arg Val Met Ser Ala Gln Leu Asn  
 340 345 350  
 Ala Ile Phe His Asp  
 355

- (2) INFORMATION FOR SEQ ID NO:3:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1275 base pairs

(B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: cDNA  
 (vi) ORIGINAL SOURCE:  
 (A) ORGANISM: *Xenopus laevis*  
 (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 25..1083  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATTTCCTTTC TCCAGATCTA AAAA ATG ACC AAA TCG TAT GGA GAG AAT GGG	51
Met Thr Lys Ser Tyr Gly Glu Asn Gly	
1 5	
CTG ATC CTG GCC GAG ACT CCG GGC TGC AGA GGA TGG GTG GAC GAA TGC	99
Leu Ile Leu Ala Glu Thr Pro Gly Cys Arg Gly Trp Val Asp Glu Cys	
10 15 20 25	
CTG AGT TCT CAG GAT GAA AAC GAT CTG GAG AAA AAG GAG GGA GAG TTG	147
Leu Ser Ser Gln Asp Glu Asn Asp Leu Glu Lys Lys Glu Gly Glu Leu	
30 35 40	
ATG AAA GAA GAC GAT GAA GAC TCA CTG AAT CAT CAC AAT GGA GAG GAG	195
Met Lys Glu Asp Asp Glu Asp Ser Leu Asn His His Asn Gly Glu Glu	
45 50 55	
AAC GAG GAA GAG GAT GAA GGG GAT GAG GAG GAG GAG GAC GAT GAA GAT	243
Asn Glu Glu Glu Asp Glu Gly Asp Glu Glu Glu Glu Asp Asp Glu Asp	
60 65 70	
GAT GAT GAG GAT GAC GAC CAG AAA CCC AAA AGG CGA GGA CCG AAA AAG	291
Asp Asp Glu Asp Asp Asp Gln Lys Pro Lys Arg Arg Gly Pro Lys Lys	
75 80 85	
AAA AAA ATG ACG AAA GCC CGG GTG GAG CGA TTT AAA GTG AGA CGC ATG	339
Lys Lys Met Thr Lys Ala Arg Val Glu Arg Phe Lys Val Arg Arg Met	
90 95 100 105	
AAG GCA AAC GCC AGG GAG AGG AAT CGC ATG CAC GGA CTC AAC GAT GCC	387
Lys Ala Asn Ala Arg Glu Arg Asn Arg Met His Gly Leu Asn Asp Ala	
110 115 120	
CTG GAC AGT CTG CGC AAA GTT GTG CCC TGC TAC TCC AAA ACA CAA AAG	435
Leu Asp Ser Leu Arg Lys Val Val Pro Cys Tyr Ser Lys Thr Gln Lys	
125 130 135	
TTG TCT AAG ATT GAA ACT CTG CGC CTG GCT AAG AAC TAC ATC TGG GCT	483
Leu Ser Lys Ile Glu Thr Leu Arg Leu Ala Lys Asn Tyr Ile Trp Ala	
140 145 150	
CTT TCT GAG ATT TTA AGG TCC GGC AAA AGC CCA GAC CTG GTG TCC TTT	531
Leu Ser Glu Ile Leu Arg Ser Gly Lys Ser Pro Asp Leu Val Ser Phe	
155 160 165	
GTA CAA ACT CTC TGC AAA GGT TTG TCG CAG CCC ACC ACC AAT CTA GTA	579
Val Gln Thr Leu Cys Lys Gly Leu Ser Gln Pro Thr Thr Asn Leu Val	
170 175 180 185	
GCG GGG TGT CTG CAG CTG AAC CCC AGA ACT TTC CTT CCT GAG CAG AGT	627
Ala Gly Cys Leu Gln Leu Asn Pro Arg Thr Phe Leu Pro Glu Gln Ser	
190 195 200	
CAG GAC ATC CAG TCG CAC ATG CAA ACA GCG AGC TCT TCC TTC CCT CTG	675
Gln Asp Ile Gln Ser His Met Gln Thr Ala Ser Ser Ser Phe Pro Leu	
205 210 215	

CAG GGC TAT CCC TAT CAG TCC CCT GGT CTT CCC AGT CCC CCC TAT GGT 723  
 Gln Gly Tyr Pro Tyr Gln Ser Pro Gly Leu Pro Ser Pro Pro Tyr Gly  
 220 225 230

ACC ATG GAC AGC TCC CAT GTA TTC CAC GTC AAG CCT CAC TCC TAT GGG 771  
 Thr Met Asp Ser Ser His Val Phe His Val Lys Pro His Ser Tyr Gly  
 235 240 245

GCG GCC CTG GAG CCT TTC TTT GAC AGC AGC ACC GTC ACT GAG TGT ACC 819  
 Ala Ala Leu Glu Pro Phe Phe Asp Ser Ser Thr Val Thr Glu Cys Thr  
 250 255 260 265

AGC CCG TCA TTC GAT GGT CCC CTG AGC CCA CCC CTT AGT GTT AAT GGG 867  
 Ser Pro Ser Phe Asp Gly Pro Leu Ser Pro Pro Leu Ser Val Asn Gly  
 270 275 280

AAC TTT ACT TTT AAA CAC GAG CAT TCG GAG TAT GAT AAA AAT TAC ACG 915  
 Asn Phe Thr Phe Lys His Glu His Ser Glu Tyr Asp Lys Asn Tyr Thr  
 285 290 295

TTC ACT ATG CAC TAT CCT GCA GCC ACT ATA TCC CAG GGC CAC GGA CCA 963  
 Phe Thr Met His Tyr Pro Ala Ala Thr Ile Ser Gln Gly His Gly Pro  
 300 305 310

TTG TTC TCC ACG GGG GGA CCA CGC TGT GAA ATC CCA ATA GAC ACC ATC 1011  
 Leu Phe Ser Thr Gly Gly Pro Arg Cys Glu Ile Pro Ile Asp Thr Ile  
 315 320 325

ATG TCC TAT GAC GGT CAC TCC CAC CAT GAA AGA GTC ATG AGT GCC CAG 1059  
 Met Ser Tyr Asp Gly His Ser His His Glu Arg Val Met Ser Ala Gln  
 330 335 340 345

CTA AAT GCC ATC TTT CAT GAT TAACCCTTGG AAGATCAAAA CAACTGACTG 1110  
 Leu Asn Ala Ile Phe His Asp  
 350

TGCATTGCCA GGACTGTCTT GTTTACCAAG GGCAGACACG TGGGTAGTAA AAGTGCAAAT 1170

GCCCCACTCT GGGGCTGTAA CAACTTGAT CTTGTCCTGC CTTTAGATAT GGGGAAACCT 1230

AATGTATTAA TTCCACCTC CTTCCAATCG ACACTCCTTT AAATT 1275

## (2) INFORMATION FOR SEQ ID NO:4:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 352 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Thr Lys Ser Tyr Gly Glu Asn Gly Leu Ile Leu Ala Glu Thr Pro  
 1 5 10 15

Gly Cys Arg Gly Trp Val Asp Glu Cys Leu Ser Ser Gln Asp Glu Asn  
 20 25 30

Asp Leu Glu Lys Lys Glu Gly Glu Leu Met Lys Glu Asp Asp Glu Asp  
 35 40 45

Ser Leu Asn His His Asn Gly Glu Glu Asn Glu Glu Asp Glu Gly  
 50 55 60

Asp Glu Glu Glu Glu Asp Asp Glu Asp Asp Asp Glu Asp Asp Asp Gln  
 65 70 75 80

Lys	Pro	Lys	Arg	Arg 85	Gly	Pro	Lys	Lys	Lys 90	Lys	Met	Thr	Lys	Ala 95	Arg
Val	Glu	Arg	Phe 100	Lys	Val	Arg	Arg	Met 105	Lys	Ala	Asn	Ala	Arg 110	Glu	Arg
Asn	Arg	Met 115	His	Gly	Leu	Asn	Asp 120	Ala	Leu	Asp	Ser	Leu 125	Arg	Lys	Val
Val	Pro 130	Cys	Tyr	Ser	Lys	Thr 135	Gln	Lys	Leu	Ser	Lys 140	Ile	Glu	Thr	Leu
Arg 145	Leu	Ala	Lys	Asn	Tyr 150	Ile	Trp	Ala	Leu	Ser 155	Glu	Ile	Leu	Arg	Ser 160
Gly	Lys	Ser	Pro	Asp 165	Leu	Val	Ser	Phe	Val 170	Gln	Thr	Leu	Cys	Lys 175	Gly
Leu	Ser	Gln	Pro 180	Thr	Thr	Asn	Leu	Val 185	Ala	Gly	Cys	Leu	Gln 190	Leu	Asn
Pro	Arg	Thr 195	Phe	Leu	Pro	Glu	Gln 200	Ser	Gln	Asp	Ile	Gln 205	Ser	His	Met
Gln	Thr 210	Ala	Ser	Ser	Ser	Phe 215	Pro	Leu	Gln	Gly	Tyr 220	Pro	Tyr	Gln	Ser
Pro 225	Gly	Leu	Pro	Ser	Pro 230	Pro	Tyr	Gly	Thr	Met 235	Asp	Ser	Ser	His	Val 240
Phe	His	Val	Lys	Pro 245	His	Ser	Tyr	Gly	Ala 250	Ala	Leu	Glu	Pro	Phe 255	Phe
Asp	Ser	Ser	Thr 260	Val	Thr	Glu	Cys	Thr 265	Ser	Pro	Ser	Phe	Asp 270	Gly	Pro
Leu	Ser	Pro 275	Pro	Leu	Ser	Val	Asn 280	Gly	Asn	Phe	Thr	Phe 285	Lys	His	Glu
His	Ser 290	Glu	Tyr	Asp	Lys	Asn 295	Tyr	Thr	Phe	Thr	Met 300	His	Tyr	Pro	Ala
Ala 305	Thr	Ile	Ser	Gln	Gly 310	His	Gly	Pro	Leu	Phe 315	Ser	Thr	Gly	Gly	Pro 320
Arg	Cys	Glu	Ile	Pro 325	Ile	Asp	Thr	Ile	Met 330	Ser	Tyr	Asp	Gly	His 335	Ser
His	His	Glu	Arg 340	Val	Met	Ser	Ala	Gln 345	Leu	Asn	Ala	Ile	Phe 350	His	Asp

(2) INFORMATION FOR SEO ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 7 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide
- (v) FRAGMENT TYPE: internal
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Asn Ala Arg Glu Arg Arg Arg  
1 5

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 7 amino acids

- (B) TYPE: amino acid  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: peptide  
 (v) FRAGMENT TYPE: internal  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Asn Glu Arg Glu Arg Asn Arg  
 1 5

## (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 5 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: peptide  
 (v) FRAGMENT TYPE: internal  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Asn Ala Arg Glu Arg  
 1 5

## (2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 524 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: DNA (genomic)  
 (vi) ORIGINAL SOURCE:  
 (A) ORGANISM: Homo sapiens  
 (vii) IMMEDIATE SOURCE:  
 (B) CLONE: 9F1  
 (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 57..524  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TTTTTCIGCT TTTCTTCTG TTTGCCTCTC CCTTGTTGAA TGTAGGAAAT CGAAAC	56
ATG ACC AAA TCG TAC AGC GAG AGT GGG CTG ATG GGC GAG CCT CAG CCC Met Thr Lys Ser Tyr Ser Glu Ser Gly Leu Met Gly Glu Pro Gln Pro	104
CAA GGT CCT CCA AGC TGG ACA GAC GAG TGT CTC AGT TCT CAG GAC GAG Gln Gly Pro Pro Ser Trp Thr Asp Glu Cys Leu Ser Ser Gln Asp Glu	152
GAG CAC GAG GCA GAC AAG AAG GAG GAC GAC CTC GAA GCC ATG AAC GCA Glu His Glu Ala Asp Lys Lys Glu Asp Asp Leu Glu Ala Met Asn Ala	200
GAG GAG GAC TCA CTG AGG AAC GGG GGA GAG GAG GAG GAC GAA GAT GAG Glu Glu Asp Ser Leu Arg Asn Gly Gly Glu Glu Glu Asp Glu Asp Glu	248
GAC CTG GAA GAG GAG GAA GAA GAG GAA GAG GAG GAT GAC GAT CAA AAG Asp Leu Glu Glu Glu Glu Glu Glu Glu Glu Asp Asp Asp Gln Lys	296
CCC AAG AGA CGC GGC CCC AAA AAG AAG AAG ATG ACT AAG GCT CGC CTG Pro Lys Arg Arg Gly Pro Lys Lys Lys Lys Met Thr Lys Ala Arg Leu	344
GAG CGT TTT AAA TTG AGA CGC ATG AAG GCT AAC GCC CGG GAG CGG AAC Glu Arg Phe Lys Leu Arg Arg Met Lys Ala Asn Ala Arg Glu Arg Asn	392

CGC ATG CAC GGA CTG AAC GCG GCG CTA GAC AAC CTG CGC AAG GTG GTG 440  
 Arg Met His Gly Leu Asn Ala Ala Leu Asp Asn Leu Arg Lys Val Val  
           115                                  120                                  125

CCT TGC TAT TCT AAG ACG CAG AAG CTG TCC AAA ATC GAG ACT CTG CGC 488  
 Pro Cys Tyr Ser Lys Thr Gln Lys Leu Ser Lys Ile Glu Thr Leu Arg  
           130                                  135                                  140

TTG GCC AAG AAC TAC ATC TGG GCT CTG TCG GAG ATC 524  
 Leu Ala Lys Asn Tyr Ile Trp Ala Leu Ser Glu Ile  
           145                                  150                                  155

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 156 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Met Thr Lys Ser Tyr Ser Glu Ser Gly Leu Met Gly Glu Pro Gln Pro  
   1                                  5                                  10                                  15

Gln Gly Pro Pro Ser Trp Thr Asp Glu Cys Leu Ser Ser Gln Asp Glu  
                                   20                                  25                                  30

Glu His Glu Ala Asp Lys Lys Glu Asp Asp Leu Glu Ala Met Asn Ala  
                                   35                                  40                                  45

Glu Glu Asp Ser Leu Arg Asn Gly Gly Glu Glu Glu Asp Glu Asp Glu  
           50                                  55                                  60

Asp Leu Glu Glu Glu Glu Glu Glu Glu Glu Asp Asp Asp Gln Lys  
           65                                  70                                  75                                  80

Pro Lys Arg Arg Gly Pro Lys Lys Lys Lys Met Thr Lys Ala Arg Leu  
                                   85                                  90                                  95

Glu Arg Phe Lys Leu Arg Arg Met Lys Ala Asn Ala Arg Glu Arg Asn  
                                   100                                  105                                  110

Arg Met His Gly Leu Asn Ala Ala Leu Asp Asn Leu Arg Lys Val Val  
           115                                  120                                  125

Pro Cys Tyr Ser Lys Thr Gln Lys Leu Ser Lys Ile Glu Thr Leu Arg  
           130                                  135                                  140

Leu Ala Lys Asn Tyr Ile Trp Ala Leu Ser Glu Ile  
           145                                  150                                  155

## (2) INFORMATION FOR SEQ ID NO:10:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1535 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(vii) IMMEDIATE SOURCE:

(B) CLONE: 14B1 (neuroD2)

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 55..1194

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```

CCCCTCACTT TGTGCTGTCT GTCTCCCCTT CCCGCCCGGG GNCCCTCAGG CACCATGCTG      60
ACCCGCCTGT TCAGCGAGCC CGGCCTTCTC TCGGACGTGC CCAAGTTCGC CAGCTGGGGC      120
GACGGCGAAG ACGACGAGCC GAGGAGCGAC AAGGGCGACG CGCCGCCACC GCCACCGCCT      180
GCGCCCGGGC CAGGGGCTCC GGGGCCAGCC CGGGCGGCCA AGCCAGTCCC TCTCCGTGGA      240
GAAGAGGGGA CGGAGGCCAC GTTGGCCGAG GTCAAGGAGG AAGGCGAGCT GGGGGGAGAG      300
GAGGAGGAGG AAGAGGAGGA GGAAGAAGGA CTGGACGAGG CGGAGGGCGA GCGGCCCAAG      360
AAGCGCGGGC CCAAGAAGCG CAAGATGACC AAGGCGCGCT TGGAGCGCTC CAAGCTTCGG      420
CGGCAGAAGG CGAACGCGCG GGAGCGCAAC CGCATGCACG ACCTGAACGC AGCCCTGGAC      480
AACCTGCGCA AGGTGGTGCC CTGCTACTCC AAGACGCAGA AGCTGTCCAA GATCGAGACG      540
CTGCGCCTAG CCAAGAACTA TATCTGGGCG CTCTCGGAGA TCCTGCGCTC CGGCAAGCGG      600
CCAGACCTAG TGTCTACGT GCAGACTCTG TGCAAGGGTC TGTGCGAGCC CACCACCAAT      660
CTGGTGGCCG GCTGTCTGCA GCTCAACTCT CGCAACTTCC TCACGGAGCA AGGCGCCGAC      720
GGTGCCGGCC GCTTCCACGG CTCGGGCGGC CCGTTCGCCA TGCACCCCTA CCCGTACCCG      780
TGCTCGCGCC TGGCGGGCGC ACAGTGCCAG GCGGCCGGCG GCCTGGGCGG CGGCGCGGCG      840
CACGCCCTGC GGACCCACGG CTACTGCGCC GCCTACGAGA CGCTGTATGC GGCGGCAGGC      900
GGTGGCGGCG CGAGCCCGGA CTACAACAGC TCCGAGTACG AGGGCCCGCT CAGCCCCCGG      960
CTCTGTCTCA ATGGCAACTT CTCACTCAAG CAGGACTCCT CGCCCGACCA CGAGAAAAGC     1020
TACCACTACT CTATGACTA CTCGGCGCTG CCCGGTTCGC GCCACGGCCA CGGGCTAGTC     1080
TTCGGCTCGT CGGCTGTGCG CGGGGGCGTC CACTCGGAGA ATCTCTTGTC TTACGATATG     1140
CACCTTCACC ACGACCGGGG CCCCATGTAC GAGGAGCTCA ATGCGTTTTT TCATAACTGA     1200
GACTTCGCGC CGNCTCCCTN CTTTTTCTTT TGCCTTTGCC CGCCCCCTG TCCCCAGCCC     1260
CCAGAGCGCA GGGACACCCC CATNCTACCC CGGCNCCGGC GGAGCGGGCC ACCGGTCTGC     1320
CGCTCTCCTG GGGCAGCGCA GTCTGTTACN TGTGGGTGGC TGTCCCAGGG GCCTCGCTTC     1380
CCCCAGGGAC TCGCCTTCTC TCTCCAAGGG GTTCCCTCCT CCTCTCTCCC AAGGAGTGCT     1440
TCTCCAGGGA CCTCTCTCCG GGGGCTCCCT GGAGGCACCC CTCCCCATT CCCAATATCT     1500
TCGCTGAGGT TTCCTCCTCC CCCTCCTCCC TGCAG                                  1535

```

## (2) INFORMATION FOR SEQ ID NO:11:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 381mino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```

Met Leu Thr Arg Leu Phe Ser Glu Pro Gly Leu Leu Ser Asp Val Pro
1           5           10           15

```



Lys Phe Ala Ser Trp Gly Asp Gly Glu Asp Asp Glu Pro Arg Ser Asp  
 20 25 30  
 Lys Gly Asp Ala Pro Pro Pro Pro Pro Pro Ala Pro Gly Pro Gly Ala  
 35 40 45  
 Pro Gly Pro Ala Arg Ala Ala Lys Pro Val Pro Leu Arg Gly Glu Glu  
 50 55 60  
 Gly Thr Glu Ala Thr Leu Ala Glu Val Lys Glu Glu Gly Glu Leu Gly  
 65 70 75 80  
 Gly Glu Glu Glu Glu Glu Glu Glu Glu Glu Glu Gly Leu Asp Glu Ala  
 85 90 95  
 Glu Gly Glu Arg Pro Lys Lys Arg Gly Pro Lys Lys Arg Lys Met Thr  
 100 105 110  
 Lys Ala Arg Leu Glu Arg Ser Lys Leu Arg Arg Gln Lys Ala Asn Ala  
 115 120 125  
 Arg Glu Arg Asn Arg Met His Asp Leu Asn Ala Ala Leu Asp Asn Leu  
 130 135 140  
 Arg Lys Val Val Pro Cys Tyr Ser Lys Thr Gln Lys Leu Ser Lys Ile  
 145 150 155 160  
 Glu Thr Leu Arg Leu Ala Lys Asn Tyr Ile Trp Ala Leu Ser Glu Ile  
 165 170 175  
 Leu Arg Ser Gly Lys Arg Pro Asp Leu Val Ser Tyr Val Gln Thr Leu  
 180 185 190  
 Cys Lys Gly Leu Ser Gln Pro Thr Thr Asn Leu Val Ala Gly Cys Leu  
 195 200 205  
 Gln Leu Asn Ser Arg Asn Phe Leu Thr Glu Gln Gly Ala Asp Gly Ala  
 210 215 220  
 Gly Arg Phe His Gly Ser Gly Gly Pro Phe Ala Met His Pro Tyr Pro  
 225 230 235 240  
 Tyr Pro Cys Ser Arg Leu Ala Gly Ala Gln Cys Gln Ala Ala Gly Gly  
 245 250 255  
 Leu Gly Gly Gly Ala Ala His Ala Leu Arg Thr His Gly Tyr Cys Ala  
 260 265 270  
 Ala Tyr Glu Thr Leu Tyr Ala Ala Ala Gly Gly Gly Gly Ala Ser Pro  
 275 280 285  
 Asp Tyr Asn Ser Ser Glu Tyr Glu Gly Pro Leu Ser Pro Pro Leu Cys  
 290 295 300  
 Leu Asn Gly Asn Phe Ser Leu Lys Gln Asp Ser Ser Pro Asp His Glu  
 305 310 315 320  
 Lys Ser Tyr His Tyr Ser Met His Tyr Ser Ala Leu Pro Gly Ser Arg  
 325 330 335  
 His Gly His Gly Leu Val Phe Gly Ser Ser Ala Val Arg Gly Gly Val  
 340 345 350  
 His Ser Glu Asn Leu Leu Ser Tyr Asp Met His Leu His His Asp Arg  
 355 360 365

Gly Pro Met Tyr Glu Glu Leu Asn Ala Phe Phe His Asn  
 370 375 380

## (2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1268 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: DNA (genomic)  
 (vi) ORIGINAL SOURCE:  
 (A) ORGANISM: Homo sapiens  
 (vii) IMMEDIATE SOURCE:  
 (B) CLONE: 20A1 (neuroD3)  
 (ix) FEATURE:  
 (A) NAME/KEY: CDS  
 (B) LOCATION: 55..768  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CTGCAGCGCT CTGAGCCGCT TTCTATCTGT CCGTCGGTCC TGCACAGCGC AACG ATG 57  
 Met  
 1

CCA GCC CGC CTT GAG ACC TGC ATC TCC GAC CTC GAC TGC GCC AGC AGC 105  
 Pro Ala Arg Leu Glu Thr Cys Ile Ser Asp Leu Asp Cys Ala Ser Ser  
 5 10 15

AGC GGC AGT GAC CTA TCC GGC TTC CTC ACC GAC GAG GAA GAC TGT GCC 153  
 Ser Gly Ser Asp Leu Ser Gly Phe Leu Thr Asp Glu Glu Asp Cys Ala  
 20 25 30

AGA CTC CAA CAG GCA GCC TCC GCT TCG GGG CCG CCC GCG CCG GCC CGC 201  
 Arg Leu Gln Gln Ala Ala Ser Ala Ser Gly Pro Pro Ala Pro Ala Arg  
 35 40 45

AGG GGC GCG CCC AAT ATC TCC CGG GCG TCT GAG GTT CCA GGG GCA CAG 249  
 Arg Gly Ala Pro Asn Ile Ser Arg Ala Ser Glu Val Pro Gly Ala Gln  
 50 55 60 65

GAC GAC GAG CAG GAG AGG CGG CGG CGC CGC GGC CGG ACG CGG GTC CGC 297  
 Asp Asp Glu Gln Arg Arg Arg Arg Arg Gly Arg Thr Arg Val Arg  
 70 75 80

TCC GAG GCG CTG CTG CAC TCG CTG CGC AGG AGC CGG CGC GTC AAG GCC 345  
 Ser Glu Ala Leu Leu His Ser Leu Arg Arg Ser Arg Arg Val Lys Ala  
 85 90 95

AAC GAT CGC GAG CGC AAC CGC ATG CAC AAC TTG AAC GCG GCC CTG GAC 393  
 Asn Asp Arg Glu Arg Asn Arg Met His Asn Leu Asn Ala Ala Leu Asp  
 100 105 110

GCA CTG CGC AGC GTG CTG CCC TCG TTC CCC GAC GAC ACC AAG CTC ACC 441  
 Ala Leu Arg Ser Val Leu Pro Ser Phe Pro Asp Asp Thr Lys Leu Thr  
 115 120 125

AAA ATC GAG ACG CTG CGC TTC GCC TAC AAC TAC ATC TGG GCT CTG GCC 489  
 Lys Ile Glu Thr Leu Arg Phe Ala Tyr Asn Tyr Ile Trp Ala Leu Ala  
 130 135 140 145

GAG ACA CTG CGC CTG GCG GAT CAA GGG CTG CCC GGA GGC GGT GCC CGG 537  
 Glu Thr Leu Arg Leu Ala Asp Gln Gly Leu Pro Gly Gly Gly Ala Arg  
 150 155 160

GAG CGC CTC CTG CCG CCG CAG TGC GTC CCC TGC CTG CCC GGT CCC CCA 585  
 Glu Arg Leu Leu Pro Pro Gln Cys Val Pro Cys Leu Pro Gly Pro Pro

165	170	175	
AGC CCC GCC AGC GAC GCG GAG TCC TGG GGC TCA GGT GCC GCC GCC GCC			633
Ser Pro Ala Ser Asp Ala Glu Ser Trp Gly Ser Gly Ala Ala Ala Ala			
180	185	190	
TCC CCG CTC TCT GAC CCC AGT AGC CCA GCC GCC TCC GAA GAC TTC ACC			681
Ser Pro Leu Ser Asp Pro Ser Ser Pro Ala Ala Ser Glu Asp Phe Thr			
195	200	205	
TAC CGC CCC GGC GAC CCT GTT TTC TCC TTC CCA AGC CTG CCC AAA GAC			729
Tyr Arg Pro Gly Asp Pro Val Phe Ser Phe Pro Ser Leu Pro Lys Asp			
210	215	220	225
TTG CTC CAC ACA ACG CCC TGT TTC ATT CCT TAC CAC TAGGCCCTTT			775
Leu Leu His Thr Thr Pro Cys Phe Ile Pro Tyr His			
230	235		
GTAGACACTG TTACTTTCCC CCTCCCCTAG TCAGCAGGCA ATAGATTGGG CCCAGCTGCC			835
GCCTCGGGAC CCCTCTCCAG GCGGAGGGAG GAAGCGGGAG CTTTAAAGCA GTCGGGGATA			895
CCTGAGCCGC TTGTTAGGTC GCCGCACCTT CGCGGCGGAT GTCTCTTGGT CTGTTTCTCC			955
GGCCCTCAGC CCAGCGCCCC TCCTGCCCCG CCCTAGACGG CCTTTCCTTT TGCACCTTCT			1015
GAACTCCACA AAACCTCCTT TGTGACTGEC TCAGAACTGA CCCCAGCCAC CACTTCAGTG			1075
TGATTTAGAA AAGGGACAGA TCAGCCCCTG AAGACGAGGT GAAAAGTCAA TTTTACAATT			1135
TGTAGAACTC TAATGAAGAA AAACGAGCAT GAAAATTCGG TTTGAGCCGG CTGACAATAC			1195
AATGAAAAGG CTTAAAAAGC AGAGACAAGG AGTGGGCTTC ATGCATTATG GATCCCGACC			1255
CCCACCACTG CAG			1268

## (2) INFORMATION FOR SEQ ID NO:13:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 237 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Met	Pro	Ala	Arg	Leu	Glu	Thr	Cys	Ile	Ser	Asp	Leu	Asp	Cys	Ala	Ser
1				5					10					15	
Ser	Ser	Gly	Ser	Asp	Leu	Ser	Gly	Phe	Leu	Thr	Asp	Glu	Glu	Asp	Cys
			20					25					30		
Ala	Arg	Leu	Gln	Gln	Ala	Ala	Ser	Ala	Ser	Gly	Pro	Pro	Ala	Pro	Ala
		35					40					45			
Arg	Arg	Gly	Ala	Pro	Asn	Ile	Ser	Arg	Ala	Ser	Glu	Val	Pro	Gly	Ala
		50				55					60				
Gln	Asp	Asp	Glu	Gln	Glu	Arg	Arg	Arg	Arg	Arg	Gly	Arg	Thr	Arg	Val
65				70					75						80
Arg	Ser	Glu	Ala	Leu	Leu	His	Ser	Leu	Arg	Arg	Ser	Arg	Arg	Val	Lys
				85				90						95	
Ala	Asn	Asp	Arg	Glu	Arg	Asn	Arg	Met	His	Asn	Leu	Asn	Ala	Ala	Leu
			100					105					110		
Asp	Ala	Leu	Arg	Ser	Val	Leu	Pro	Ser	Phe	Pro	Asp	Asp	Thr	Lys	Leu

115	120	125
Thr Lys Ile Glu Thr Leu Arg Phe Ala Tyr Asn Tyr Ile Trp Ala Leu		
130	135	140
Ala Glu Thr Leu Arg Leu Ala Asp Gln Gly Leu Pro Gly Gly Gly Ala		
145	150	155
Arg Glu Arg Leu Leu Pro Pro Gln Cys Val Pro Cys Leu Pro Gly Pro		
165	170	175
Pro Ser Pro Ala Ser Asp Ala Glu Ser Trp Gly Ser Gly Ala Ala Ala		
180	185	190
Ala Ser Pro Leu Ser Asp Pro Ser Ser Pro Ala Ala Ser Glu Asp Phe		
195	200	205
Thr Tyr Arg Pro Gly Asp Pro Val Phe Ser Phe Pro Ser Leu Pro Lys		
210	215	220
Asp Leu Leu His Thr Thr Pro Cys Phe Ile Pro Tyr His		
225	230	235

## (2) INFORMATION FOR SEQ ID NO:14:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1560 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(vii) IMMEDIATE SOURCE:

(B) CLONE: HC2A

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 57..1126

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TTTTTCTGCT TTTCTTTCTG TTTGCCTCTC CCTTGTGAA TGTAGGAAAT CGAAACATGA	60
CCAAATCGTA CAGCGAGAGT GGGCTGATGG GCGAGCCTCA GCCCCAAGGT CCTCCAAGCT	120
GGACAGACGA GTGTCTCAGT TCTCAGGACG AGGAGCACGA GGCAGACAAG AAGGAGGACG	180
ACCTCGAAGC CATGAACGCA GAGGAGGACT CACTGAGGAA CGGGGGAGAG GAGGAGGACG	240
AAGATGAGGA CCTGGAAGAG GAGGAAGAAG AGGAAGAGGA GGATGACGAT CAAAAGCCCA	300
AGAGACGCGG CCCCCAAAAG AAGAAGATGA CTAAGGCTCG CCTGGAGCGT TTAAATTGA	360
GACGCATGAA GGCTAACGCC CGGGAGCGGA ACCGCATGCA CGGACTGAAC GCGGCGCTAG	420
ACAACCTGCG CAAGGTGGTG CCTTGCTATT CTAAGACGCA GAAGCTGTCC AAAATCGAGA	480
CTCTGCGCTT GGCCAAGAAC TACATCTGGG CTCTGTCGGA GATCCTGCGC TCAGGCAAAA	540
GCCCAGACCT GGTCTCCTTC GTTCAGACGC TTTGCAAGGG CTTATCCCAA CCCACCACCA	600
ACCTGGTTGC GGGCTGCCTG CAACTCAATC CTCGGACTTT TCTGCCTGAG CAGAACCAGG	660
ACATGCCCCC GCACCTGCCG ACGGCCAGCG CTTCTTCCC TGTACACCCC TACTCCTACC	720
AGTCGCCTGG GCTGCCAGT CCGNCTTACG GTACCATGGA CAGCTCCCAT GTCTTCCACG	780
TTAAGCCTCC GCCGCACGCC TACAGCGCAG CGCTGGAGCC CTTCTTTGAA AGCCCTCTGA	840

```

CTGATTGCAC CAGCCCTTCC TTTGATGGAC CCCTCAGCCC GCCGCTCAGC ATCAATGGCA      900
ACTTCTCTTT CAAACACGAA CCGTCCGCCG AGTTTGAGAA AAATTATGCC TTTACCATGC      960
ACTATCCTGC AGCGACACTG GCAGGGGCCC AAAGCCACGG ATCAATCTTC TCAGGCACCG      1020
CTGCCCCTCG CTGCGAGATC CCCATAGACA ATATTATGTC CTTCGATAGC CATTACATC      1080
ATGAGCGAGT CATGAGTGCC CAGCTCAATG CCATATTTCA TGATTAGAGG CACGCCAGTT      1140
TCACCATTTC CGGGAAACGA ACCCACTGTG CTTACAGTGA CTGTCGTGTT TACAAAAGGC      1200
AGCCCTTTGG TACTACTGCT GCAAAGTGCA AATACTCCAA GCTTCAAGTG ATATATGTAT      1260
TTATTGTCAT TACTGCCTTT GGAAGAAACA GGGGATCAAA GTTCCTGTTC ACCTTATGTA      1320
TTATTTTCTA TAGACTCTTC TATTTTAAAA AATAAAAAAA TACAGTAAAG TTTAAAAAAT      1380
ACACCACGAA TTTGGTGTGG CTGTATTCAG ATCGTATTAA TTATCTGATC GGGATAACAA      1440
AATCACAAGC AATAATTAGG ATCTATGCAA TTTTAAACT AGTAATGGGC CAATTAAAAT      1500
ATATATAAAT ATATATTTCA ACCAGCATTT TACTACTTGT TACCTCCCAT GCTGAATTAT      1560

```

## (2) INFORMATION FOR SEQ ID NO:15:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 356 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Homo sapiens

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```

Met Thr Lys Ser Tyr Ser Glu Ser Gly Leu Met Gly Glu Pro Gln Pro
 1           5           10           15
Gln Gly Pro Pro Ser Trp Thr Asp Glu Cys Leu Ser Ser Gln Asp Glu
          20           25           30
Glu His Glu Ala Asp Lys Lys Glu Asp Asp Leu Glu Ala Met Asn Ala
          35           40           45
Glu Glu Asp Ser Leu Arg Asn Gly Gly Glu Glu Glu Asp Glu Asp Glu
          50           55           60
Asp Leu Glu Glu Glu Glu Glu Glu Glu Glu Asp Asp Asp Gln Lys
65           70           75           80
Pro Lys Arg Arg Gly Pro Lys Lys Lys Lys Met Thr Lys Ala Arg Leu
          85           90           95
Glu Arg Phe Lys Leu Arg Arg Met Lys Ala Asn Ala Arg Glu Arg Asn
          100          105          110
Arg Met His Gly Leu Asn Ala Ala Leu Asp Asn Leu Arg Lys Val Val
          115          120          125
Pro Cys Tyr Ser Lys Thr Gln Lys Leu Ser Lys Ile Glu Thr Leu Arg
          130          135          140
Leu Ala Lys Asn Tyr Ile Trp Ala Leu Ser Glu Ile Leu Arg Ser Gly
          145          150          155          160
Lys Ser Pro Asp Leu Val Ser Phe Val Gln Thr Leu Cys Lys Gly Leu

```

[illegible]

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1951 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: *Mus musculus*

(vii) IMMEDIATE SOURCE:

(B) CLONE: 1.1.1 (mouse neuroD2)

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 230..1378

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GAATTCAAGC	TAGAGGCTGG	TACCCCGCCT	GGTAGAGATG	CCACACTCGC	TCCGCGGCTC	60
GCATGGCGCT	CTGAAGACGC	CGGCGCCCGC	GCCTTGAGGA	ACCGCTGCCC	CCGCTCCCTG	120
AAGATGGGGG	AACAATGAAA	TAAGCGAGAA	GATTCTCTTT	CTCCCCCCTC	TCTCTCTTGC	180
CCCCTCCCCC	CTCCCCTCCC	CTCTCCCCTT	GACTCTCTCT	TGAGGCACCA	TGCTGACCCG	240
CCTGTTTACG	GAGCCCGGCC	TCCTCTCGGA	CGTGCCCAAG	TTCGCCAGCT	GGGGCGACGG	300
CGACGACGAC	GAGCCGAGGA	GCGACAAGGG	CGACGCGCCG	CCGCAGCCTT	CTCCTGCTCC	360

CGGGTCGGGG GCTCCAGGAC CCGCCCGGGC CGCCAAGCCA GTGTCTCTTC GTGGAGGAGA 420  
 AGAGATCCCT GAACCCACGT TGGCTGAGGT CAAGGAGGAA GGAGAGCTGG GCGGCGAGGA 480  
 GGAGGAGGAA GAGGAGGAGG AGGAAGGACT GGACGAGGCG GAAGGCGAGC GGCCCAAGAA 540  
 GCGCGGGCCG AAGAAACGCA AGATGACCAA GGCGCTCTG GAGCGCTCCA AGCTGCGGCG 600  
 ACAGAAGGCC AATGCGCGCG AGCGCAACCG CATGCACGAC CTGAACGCGG CTCTGGACAA 660  
 CCTGCGCAAG GTGGTCCCCT GCTACTCCAA GACCCAGAAG CTGTCCAAGA TCGAGACCCT 720  
 GCGCCTGGCC AAGAACTACA TCTGGGCTCT CTCGGAGATC TTGCGCTCCG GGAAGCGGCC 780  
 GGATCTGGTG TCCTACGTGC AGACTCTGTG CAAGGGGCTG TCACAGCCCA CCACGAATCT 840  
 GGTGGCCGGC TGCCTGCAGT TAAACTCTCG TAACTTCCTC ACGGAGCAGG GCGCGGACGG 900  
 CGCCGGCCGC TTTCACGGCT CGGGTGGCCC GTTCGCCATG CATCCGTACC CATACCCGTG 960  
 CTCCCGCCTG GCAGGCGCAC AGTGTGAGG GGTGGCGGC CTGGGCGGAG GCGCGGCGCA 1020  
 CGCCCTGCGG ACCCACGGCT ACTGCGCCGC CTACGAGACG CTGTACGCGG CGGCCGGTGG 1080  
 CGGCGGCGCT AGCCCGGACT ACAACAGCTC CGAGTACGAG GGTCCACTCA GTCCCCCGCT 1140  
 CTGTCTCAAC GGCAACTTCT CGCTCAAGCA GGA CTCTGCTC CCGATCACG AGAAGAGCTA 1200  
 CCACTACTCT ATGCACTACT CGGCGCTGCC CGGCTCACGC CACGGCCACG GGCTGGTCTT 1260  
 CGGCTCGTCG GCCGTGCGCG GGGGCGTCCA CTCCGAGAAT CTCTTGCTT ACGATATGCA 1320  
 CCTTCACCAC GATCGGGGCC CCATGTACGA GGAGCTCAAC GCATTTTTC ATAAGTAGA 1380  
 CCTCNCGCCG ACCCCTTCTT TTTCTTTGCC TTTGTCCGGC CCCTTAGCCC CAGCCCCANN 1440  
 AGCTCAGGGA GCTCCCACCG ACTCCAGAGC CGGGCNCCTG NCNCGCCGCC GGTCTGCGAG 1500  
 CTCTCCAGAG CGGCGTGCTC TCTTACCTGT GGGTGGCCCC TCCCAGGGGC CTCGCTTGCC 1560  
 TCTGGGGACT CGCCTTCTCT CTCTCCCCAG CGGCTTCCTC CTCCCTTCTC TCGTGGAGAG 1620  
 CATCTCTNNN GATCTCCCGC CAGCCCTCCC AAGAGACTTC CTCCACATTC CCAAAGTTGG 1680  
 GTTTTCTCTC CCCACCTCCA ACAGGCCAGA GGAGTTGGTA AGGGGTGCTG AGTCTCGGGA 1740  
 TAGTGTCTCC CCACTTATAG TTACTTAAAC AAACAAACAG ACACAGAGCT TCCAGCNAAA 1800  
 AGAGTTGGTA TCTCTTCCTT CTCGAAGANC ACCAGCCAGG AGCCCAACCG CCTTCACCCT 1860  
 AACACNGAAT CTCCNNGTTT TTTATTTTTT ATTTTGGTGG GAGGGGATGT GGATTGAGAG 1920  
 GAAAGAGAGA GCCAAGCCAA TTTGTAAC TA G 1951

## (2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 382 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Mus musculus
- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: 1.1.1 (murine neuroD2)
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Met Leu Thr Arg Leu Phe Ser Glu Pro Gly Leu Leu Ser Asp Val Pro  
 1 5 10 15  
 Lys Phe Ala Ser Trp Gly Asp Gly Asp Asp Glu Pro Arg Ser Asp  
 20 25 30  
 Lys Gly Asp Ala Pro Pro Gln Pro Ser Pro Ala Pro Gly Ser Gly Ala  
 35 40 45  
 Pro Gly Pro Ala Arg Ala Ala Lys Pro Val Ser Leu Arg Gly Gly Glu  
 50 55 60  
 Glu Ile Pro Glu Pro Thr Leu Ala Glu Val Lys Glu Glu Gly Glu Leu  
 65 70 75 80  
 Gly Gly Glu Glu Glu Glu Glu Glu Glu Glu Glu Gly Leu Asp Glu  
 85 90 95  
 Ala Glu Gly Glu Arg Pro Lys Lys Arg Gly Pro Lys Lys Arg Lys Met  
 100 105 110  
 Thr Lys Ala Arg Leu Glu Arg Ser Lys Leu Arg Arg Gln Lys Ala Asn  
 115 120 125  
 Ala Arg Glu Arg Asn Arg Met His Asp Leu Asn Ala Ala Leu Asp Asn  
 130 135 140  
 Leu Arg Lys Val Val Pro Cys Tyr Ser Lys Thr Gln Lys Leu Ser Lys  
 145 150 155 160  
 Ile Glu Thr Leu Arg Leu Ala Lys Asn Tyr Ile Trp Ala Leu Ser Glu  
 165 170 175  
 Ile Leu Arg Ser Gly Lys Arg Pro Asp Leu Val Ser Tyr Val Gln Thr  
 180 185 190  
 Leu Cys Lys Gly Leu Ser Gln Pro Thr Thr Asn Leu Val Ala Gly Cys  
 195 200 205  
 Leu Gln Leu Asn Ser Arg Asn Phe Leu Thr Glu Gln Gly Ala Asp Gly  
 210 215 220  
 Ala Gly Arg Phe His Gly Ser Gly Gly Pro Phe Ala Met His Pro Tyr  
 225 230 235 240  
 Pro Tyr Pro Cys Ser Arg Leu Ala Gly Ala Gln Cys Gln Ala Ala Gly  
 245 250 255  
 Gly Leu Gly Gly Gly Ala Ala His Ala Leu Arg Thr His Gly Tyr Cys  
 260 265 270  
 Ala Ala Tyr Glu Thr Leu Tyr Ala Ala Ala Gly Gly Gly Gly Ala Ser  
 275 280 285  
 Pro Asp Tyr Asn Ser Ser Glu Tyr Glu Gly Pro Leu Ser Pro Pro Leu  
 290 295 300  
 Cys Leu Asn Gly Asn Phe Ser Leu Lys Gln Asp Ser Ser Pro Asp His  
 305 310 315 320  
 Glu Lys Ser Tyr His Tyr Ser Met His Tyr Ser Ala Leu Pro Gly Ser  
 325 330 335  
 Arg His Gly His Gly Leu Val Phe Gly Ser Ser Ala Val Arg Gly Gly  
 340 345 350



Val His Ser Glu Asn Leu Leu Ser Tyr Asp Met His Leu His His Asp  
355 360 365

Arg Gly Pro Met Tyr Glu Glu Leu Asn Ala Phe Phe His Asn  
370 375 380

## (2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 20 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: JL34
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CTCAGCATCA GCAACTCGGC

20

## (2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 29 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: JL36
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TCGGATCCCG TTCTAGGCGC GCCTTGGTC

29

## (2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: JL40
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GTTTTCCCAG TCACGACGTT G

21

## (2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 1333 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: double
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Mus musculus
- (vii) IMMEDIATE SOURCE:
  - (B) CLONE: neuroD3
- (ix) FEATURE:
  - (A) NAME/KEY: CDS
  - (B) LOCATION: 101..835
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CTGCAGAGGA CAGGTAGCCC CGGGTCGTAC GGACAGTAAG TGCGCTTCGA AGGCCGACCT

60

CCAAACCTCC TGTCCGTCTG TCGGTCCTGC AACTGCAAG ATG CCT GCC CCT TTG

115

	Met	Pro	Ala	Pro	Leu	
	1				5	
GAG ACC TGC ATC TCT GAT CTC GAC TGC TCC AGC AGC AAC AGC AGC AGC						163
Glu Thr Cys Ile Ser Asp Leu Asp Cys Ser Ser Ser Asn Ser Ser Ser						
	10				20	
GAC CTG TCC AGC TTC CTC ACC GAC GAG GAG GAC TGT GCC AGG CTA CAG						211
Asp Leu Ser Ser Phe Leu Thr Asp Glu Glu Asp Cys Ala Arg Leu Gln						
	25				35	
CCC CTA GCC TCC ACC TCG GGG CTG TCC GTG CCA GCC CGG AGG AGC GCT						259
Pro Leu Ala Ser Thr Ser Gly Leu Ser Val Pro Ala Arg Arg Ser Ala						
	40				50	
CCC GCC CTC TCC GGG GCA TCG AAT GTT CCC GGT GCC CAG GAC GAA GAG						307
Pro Ala Leu Ser Gly Ala Ser Asn Val Pro Gly Ala Gln Asp Glu Glu						
	55				65	
CAG GAA CGG CGG AGG CGG CGA GGT CGC GCT CGG GTG CGG TCC GAG GCT						355
Gln Glu Arg Arg Arg Arg Gly Arg Ala Arg Val Arg Ser Glu Ala						
	70				80	85
CTG CTG CAC TCC CTG CGG AGG AGT CGT CGC GTC AAA GCC AAC GAT CGC						403
Leu Leu His Ser Leu Arg Arg Ser Arg Arg Val Lys Ala Asn Asp Arg						
	90				95	100
GAG CGC AAC CGC ATG CAC AAC CTC AAC GCT GCG CTG GAC GCC TTG CGC						451
Glu Arg Asn Arg Met His Asn Leu Asn Ala Ala Leu Asp Ala Leu Arg						
	105				110	115
AGC GTG CTG CCC TCG TTC CCC GAC GAC ACC AAG CTC ACC AAG ATT GAG						499
Ser Val Leu Pro Ser Phe Pro Asp Asp Thr Lys Leu Thr Lys Ile Glu						
	120				125	130
ACG CTG CGC TTC GCC TAC AAC TAC ATC TGG GCC CTG GCT GAG ACA CTG						547
Thr Leu Arg Phe Ala Tyr Asn Tyr Ile Trp Ala Leu Ala Glu Thr Leu						
	135				140	145
CGC CTG GCA GAT CAA GGG CTC CCC GGG GGC AGT GCC CGG GAG CGC CTC						595
Arg Leu Ala Asp Gln Gly Leu Pro Gly Gly Ser Ala Arg Glu Arg Leu						
	150				155	160
CTG CCT CCG CAG TGT GTC CCC TGT CTG CCC GGG CCC CCG AGC CCG GCC						643
Leu Pro Pro Gln Cys Val Pro Cys Leu Pro Gly Pro Pro Ser Pro Ala						
	170				175	180
AGC GAC ACT GAG TCC TGG GGT TCC GGG GCC GCT GCC TCC CCC TGC GCC						691
Ser Asp Thr Glu Ser Trp Gly Ser Gly Ala Ala Ala Ser Pro Cys Ala						
	185				190	195
ACT GTG GCA TCA CCA CTC TCT GAC CCC AGT AGT CCC TCG GCT TCA GAA						739
Thr Val Ala Ser Pro Leu Ser Asp Pro Ser Ser Pro Ser Ala Ser Glu						
	200				205	210
GAC TTC ACC TAT GGC CCG GGC GAT CCC CTT TTC TCC TTT CCT GGC CTG						787
Asp Phe Thr Tyr Gly Pro Gly Asp Pro Leu Phe Ser Phe Pro Gly Leu						
	215				220	225
CCC AAA GAC CTG CTC CAC ACG ACG CCC TGT TTC ATC CCA TAC CAC TAGGCCTTTG						
842						
Pro Lys Asp Leu Leu His Thr Thr Pro Cys Phe Ile Pro Tyr His						
	230				235	240
						245
TAAGGCAACA TCAATACATT CTTCTCCCC CAGTCTAAGA GCAATAATAG ATGGGGAACT						902

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GGCTGAAGCC TCCGGGGGCC ACACTTACCC CCAAGTGAAT TCTGGGAGCT TTAAAGGGGG      962
GAGGGGGAAT ACCTGACCAC TTGTTAGGTT GCTGCACCCT CGCTGAAGCT GCCCTCGGTC      1022
TATTTCTCCA CCCCCAGCAC GGCCTCCCCC CCCCCGCCC GCCCCCAGAC GGCCTTTCGT      1082
TTTTGTTGCA CTTTCTGAAC TTCACAAAAC CTTCTTTGTG ACTGGCTCAG AACTGACCCC      1142
AGCCACCACT TCAGTGTGGT TTGGAAAAGG GACAGATGAG CCCCTGAAGA CGAGGTGAAA      1202
AGTCAATTTT ACAATTTGTA GAACTCTAAT GAAGAAAAAC GAGCATGAAA ATTCGGTTTG      1262
AGCCGGCTGA CAATACAATG GCAAGGCTTA AAAAGGAGCC ACAAGGAGTG GGCTTCATGC      1322
ATTATGGATC C                                                                1333

```

## (2) INFORMATION FOR SEQ ID NO:22:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 244 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```

Met Pro Ala Pro Leu Glu Thr Cys Ile Ser Asp Leu Asp Cys Ser Ser
 1             5             10             15
Ser Asn Ser Ser Ser Asp Leu Ser Ser Phe Leu Thr Asp Glu Glu Asp
          20             25             30
Cys Ala Arg Leu Gln Pro Leu Ala Ser Thr Ser Gly Leu Ser Val Pro
          35             40             45
Ala Arg Arg Ser Ala Pro Ala Leu Ser Gly Ala Ser Asn Val Pro Gly
          50             55             60
Ala Gln Asp Glu Glu Gln Glu Arg Arg Arg Arg Arg Gly Arg Ala Arg
          65             70             75             80
Val Arg Ser Glu Ala Leu Leu His Ser Leu Arg Arg Ser Arg Arg Val
          85             90             95
Lys Ala Asn Asp Arg Glu Arg Asn Arg Met His Asn Leu Asn Ala Ala
          100            105            110
Leu Asp Ala Leu Arg Ser Val Leu Pro Ser Phe Pro Asp Asp Thr Lys
          115            120            125
Leu Thr Lys Ile Glu Thr Leu Arg Phe Ala Tyr Asn Tyr Ile Trp Ala
          130            135            140
Leu Ala Glu Thr Leu Arg Leu Ala Asp Gln Gly Leu Pro Gly Gly Ser
          145            150            155            160
Ala Arg Glu Arg Leu Leu Pro Pro Gln Cys Val Pro Cys Leu Pro Gly
          165            170            175
Pro Pro Ser Pro Ala Ser Asp Thr Glu Ser Trp Gly Ser Gly Ala Ala
          180            185            190
Ala Ser Pro Cys Ala Thr Val Ala Ser Pro Leu Ser Asp Pro Ser Ser
          195            200            205
Pro Ser Ala Ser Glu Asp Phe Thr Tyr Gly Pro Gly Asp Pro Leu Phe
          210            215            220

```

Ser Phe Pro Gly Leu Pro Lys Asp Leu Leu His Thr Thr Pro Cys Phe  
225 230 235 240

Ile Pro Tyr His

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 bases
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GTCACAAGTC AGCGCCCAAG

20

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 nucleotides
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: oligonucleotide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CGACGAGTAG GATGAGACCG

20

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/16417**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : C07H 21/04; C12P 21/02; C12Q 1/04, 1/68

US CL : 435/6, 34, 69.1; 536/23.1

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 29, 34, 69.1, 69.4, 172.3, 257.33, 320.1, 325, 357, 360; 536/23.1, 23.5, 23.51

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, EMBASE, MEDLINE, DERWENT

search terms: neuroectodermal?, basic helix loop, tumor? or tumour?, neurod?, medulloblastoma?

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,352,595 A (TAPSCOTT et al.) 04 October 1994, see entire document.	1-6
A, P	US 5,695,995 A (WEINTRAUB et al.) 09 December 1997, see entire document.	1-6

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*E* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*g* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

22 SEPTEMBER 1998

Date of mailing of the international search report

19 OCT 1998

Name and mailing address of the ISA/US  
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